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Award Number: DAMD17-00-1-0376

TITLE: Organic Isothiocyanates: Dietary Modulators
of Doxorubicin Resistance in Breast Cancer

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REPORT DATE: June 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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|--|---|--|---|--|---|--|
| 1. AGENCY USE ONLY (Leave blank) | | | 2. REPORT DATE June 2004 | | 3. REPORT TYPE AND DATES COVERED Final (1 Jun 00-31 May 04) | |
| 4. TITLE AND SUBTITLE Organic Isothiocyanates: Dietary Modulators of Doxorubicin Resistance in Breast Cancer | | | 5. FUNDING NUMBERS DAMD17-00-1-0376 | | | |
| 6. AUTHOR(S) Marilyn E. Morris, Ph.D. | | | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) State University of New York at Buffalo Amherst, New York 14228 E-Mail: memorris@acsu.buffalo.edu | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | | | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | 10. SPONSORING / MONITORING AGENCY REPORT NUMBER | | | |
| 11. SUPPLEMENTARY NOTES Original contains color plates. All DTIC reproductions will be in black and white. | | | | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200 Words) Drug resistance is the main cause for therapeutic failure and death in breast cancer. Our goal is to evaluate dietary organic isothiocyanates (ITCs) as inhibitors of MDR. Our studies have demonstrated that phenethyl ITC (PEITC), benzyl ITC (BITC) and naphthyl ITC (NITC) can inhibit P-glycoprotein-, Multidrug Resistance-associated protein (MRP1)- and Breast Cancer Resistance Protein (BCRP)-mediated efflux, in cell lines that overexpress these transport proteins. Studies evaluating the mechanism of this interaction have suggested that PEITC is an inhibitor, but not a substrate for P-gp. PEITC represents a substrate for BCRP, and alters MRP1-mediated transport through binding interactions, as well as depletion of the cofactor for transport, glutathione. HPLC assays have been developed to determine the concentrations of these ITCs in biological samples, and a novel LC/MS/MS assay developed for PEITC, in order to obtain the needed specificity and sensitivity for in vivo studies. The stability and pharmacokinetics of NITC and PEITC have been determined for the first time. These studies represent the first report of inhibition of the ABC efflux proteins, P-glycoprotein, MRP1 and BCRP, which are important determinants of MDR. The ITCs may represent a new class of inhibitors of MDR in breast cancer. | | | | | | |
| 14. SUBJECT TERMS Drug resistance, P-glycoprotein, Doxorubicin, breast cancer, organic isothiocyanates, pharmacokinetics, drug delivery | | | | | 15. NUMBER OF PAGES 207 | |
| | | | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited | | | |

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Final Research Report June 30, 2004
DAMD170010376 Organic Isothiocyanates: Dietary Modulators of Doxorubicin
Resistance in Breast Cancer
P.I.: Marilyn E. Morris, Ph.D.

INTRODUCTION

Drug resistance is the main cause for therapeutic failure and death in breast cancer. An important mechanism of this resistance is the enhanced cellular efflux of a wide variety of structurally distinct classes of chemotherapeutic agents due to the overexpression of *P-glycoprotein (P-gp)*. In a recent study, Buser et al. (1997) reported a high prevalence of *P-gp* in breast cancer tumor tissue: 83% in early breast cancer and 100% in primarily metastatic breast cancer. One strategy for reversing *P-gp*-mediated multidrug resistance (MDR) in breast cancer has been the concomitant use of chemical agents that are by themselves nontoxic but that potentiate the accumulation of chemotherapeutic drugs in MDR cells. Current attempts to reverse MDR with inhibitors have been largely unsuccessful due to the dose-limiting cytotoxicity of the inhibitors, and due to toxicity produced as a result of the altered pharmacokinetics of the chemotherapeutic agents. We propose the use of a new class of drugs, the organic isothiocyanates (ITCs), as inhibitors of *P-gp* -mediated doxorubicin (DOX) resistance in breast cancer. The organic ITCs are components present in the diet, especially in cruciferous vegetables such as broccoli, watercress, cabbage and brussel sprouts. These compounds are of considerable interest since they have chemoprotective properties; they are potent inhibitors of enzymes involved in carcinogen activation and inducers of enzymes involved in carcinogen detoxification. We have found that three organic ITCs, phenethyl, benzyl and naphthyl ITCs, can increase the accumulation of daunomycin in the drug-resistant human breast cancer cell line MCF-7, without affecting accumulation in sensitive MCF-7 cells. We are particularly interested in these compounds as *P-gp* inhibitors first because of their chemoprotective properties and secondly because they have been shown to be nontoxic in all studies to date. Our hypothesis is that these dietary ITCs, by inhibiting *P-gp* will reverse the tumor resistance to DOX, resulting in increased efficacy in breast cancer treatment, without increasing toxicity.

BODY

Statement of Work

Task 1. Determine the concentration-dependent effect of BITC, PEITC and NITC on the accumulation of ^3H -daunomycin in sensitive and resistant MCF-7 and SK-BR-3 human breast cancer cells, and in the porcine kidney cell line LLC-PK₁.

Overview

Studies were performed to evaluate the effect of ITCs on two P-gp substrates, daunomycin and vinblastine in MCF-7 and LLC-PK1 cells. Further studies evaluated effects on another MDR efflux protein, Multidrug Resistance Protein 1 (MRP1) in human pancreatic cancer cells Panc-1.

A manuscript was published (Tseng et al, Pharm Res 19:1509-1515, 2002, present in the Appendix) and the abstract is reproduced below.

Effect of Organic Isothiocyanates on the P-glycoprotein- and MRP1-mediated Transport of Daunomycin and Vinblastine.

Purpose. Organic isothiocyanates (ITCs) (mustard oils) are non-nutrient components present in the diet, especially in cruciferous vegetables. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and Multidrug Resistance-Associated Protein (MRP1)-mediated transport in multidrug resistant (MDR) human cancer cell lines.

Methods. The direct effect of organic isothiocyanates on the 2-hour cellular accumulation of daunomycin (DNM) and vinblastine (VBL), substrates for both P-gp and MRP1, were measured in sensitive and resistant MCF-7 cells and in PANC-1 cells. Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp while PANC-1 cells overexpress MRP1. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-, phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, phenylbutyl- isothiocyanate, sulforaphane, erucin and erysolin.

Results. NITC significantly increased the accumulation of DNM and VBL in both resistant cell lines, but had no effect on DNM accumulation in sensitive MCF-7 cells. VBL accumulation in resistant MCF-7 cells was increased 40-fold by NITC, while that in PANC-1 cells was increased 5.5-fold. Significant effects on the accumulation of DNM and VBL in resistant MCF-7 cells were also observed with BITC, while PEITC, erysolin, phenylhexyl-ITC and phenylbutyl-ITC increased the accumulation of DNM and/or VBL in PANC-1 cells. Overall, the inhibitory activities of these compounds in MCF-7 cells and PANC-1 cells were significantly correlated ($r^2 = 0.77$ and 0.86 for DNM and VBL, respectively). Significant effects on accumulation were generally observed with the ITCs at 50 μ M concentrations, but not at 10 μ M concentrations.

Conclusions. One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP1-mediated efflux of DNM and VBL in MDR cancer cells, and suggest the potential for diet-drug interactions.

Task 2. Synthesize ^{14}C -labelled NITC, BITC and PEITC. The procedure for the synthesis of ^{14}C -PEITC is that used by Conaway et al (1999) and described in the grant proposal. We will begin with the synthesis of radiolabeled PEITC using this method.

Overview:

[^{14}C]Phenethyl-isothiocyanate has been prepared by a three-step synthesis, as previously described (Conaway et al., 1999; Xu and Thornalley, 1999). The starting material was [^{14}C] KCN (25mCi), and the two intermediates were [^{14}C] benzylcyanate and [^{14}C] phenethylamine. Further purification was done using a silica column and hexane 10 ml. One-ml fractions were collected and fractions 2-4 combined and the hexane evaporated under a stream of dry nitrogen. An oily liquid was obtained. NMR and HPLC methods were used to identify the product and determine the purity. The total yield, based on radioactivity, was 28%. NMR confirmed the chemical identification, and the radio-chemical purity, based on HPLC, was 99.3%.

Since the Department had obtained an LC/MS/MS instrument (PE Sciex API 3000), we investigated the use of this technique as an alternative to the use of radiolabelled compounds. We

had indicated in our original proposal that we would use LC/MS/MS if the instrumentation became available.

Task 3. Set up HPLC assays for NITC, BITC and PEITC. Stability of samples is a concern and will be addressed. Synthesize the mercapturic acid conjugates (the major metabolites) of PEITC and BITC.

Overview: We have developed HPLC assays for PEITC, BITC and NITC, and have performed stability studies for all three compounds. Since the major metabolites of PEITC are not commercially available, we synthesized and purified the mercapturic acid conjugates of PEITC and BITC, the major metabolites of PEITC and BITC in rats and humans.

a) PEITC

HPLC Assay and Stability of PEITC and PEITC Mercapturic Acid Conjugate.

Abstract.

Purpose. Phenethyl isothiocyanate (PEITC) is a component present in cruciferous vegetables that has cancer chemopreventive properties. Our objectives were to develop an HPLC assay for PEITC and its mercapturic acid conjugate, and to determine the pH-and temperature-dependent stability of PEITC. The assay was used to evaluate PEITC and its mercapturic acid conjugate in urine samples from subjects following watercress ingestion. **Methods.** The HPLC mobile phase for PEITC and its metabolite were methanol:water (60:40, v/v) and acetonitrile:10mM phosphate buffer pH 3.0 (30:70, v/v), respectively. Propiophenone was used as the internal standard. PEITC mercapturic acid was synthesized based on the method of Adesida, et al., 1996 and Brusewitz, et al., 1977. The limit of quantitation of PEITC and its mercapturic acid conjugate was 4 μ M . Stability of PEITC was studied in universal buffers of citrate-phosphate-borate/HCl at pH values of 3.0, 5.0, 7.4, 8.4, 9.4 and 10.4. All samples were studied following incubation at RT and at refrigerated temperatures. Four healthy subjects ingested 100g of fresh watercress and urine samples were collected over time and analyzed for PEITC and PEITC mercapturic acid conjugate. **Results.** PEITC degrades with first order kinetics in buffer solutions. The half-life of PEITC in a buffer of pH 7.4 at RT is 56.1 hours and at 4 °C is 108.1 hours. Urine samples obtained after watercress ingestion contained only the PEITC mercapturic acid conjugate, with greatest amounts appearing between 2-4 hours. The minimum value for bioavailability, based on urinary recovery of the mercapturic acid conjugate of PEITC, was variable with a geometric mean of 44.5% and a median value of 52.5%. **Conclusions.** An HPLC assay for PEITC and its mercapturic acid conjugate has been developed. The PEITC and its metabolite are stable under the sample collection and assay conditions.

Stability of PEITC. A universal buffer of citrate-phosphate-borate/HCl was used in this study. The buffer solutions, with pH of 2.6, 6.7 and 10.7, were spiked with a 0.1mM PEITC (in DMSO). All samples were left at room temperature and injected at various times throughout a 20-hour period. Injection volume was 50 μ l. PEITC in a buffer of pH 10.7 was found to degrade following first order kinetics, and had a half-life of 5.0 hours. PEITC in buffers of pH 2.6 and 6.7 were stable over a 20-hour period. PEITC half-lives at buffer pH's of 2.6 and 6.7 were 37.1 and 35.7 hours, respectively.

Further studies examining the stabilities of PEITC, BITC and NITC are presented in our manuscript Hu and Morris, J Pharm Sci, 93: 1901-1911, 2004, present in the Appendix.

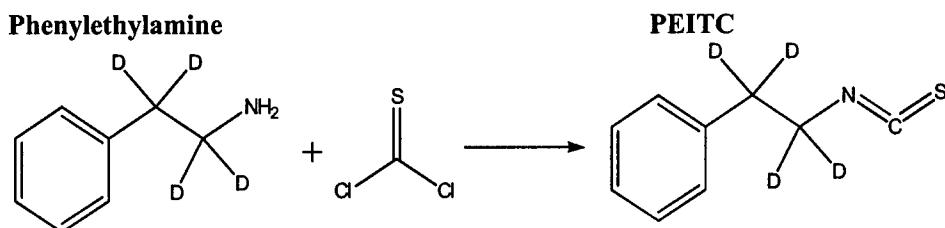
Synthesis and characterization of the mercapturic acid conjugates of PEITC and BITC.

The synthesis was base on the procedures described by Brusewitz et al. (Brusewitz et al., 1977) but with some modification. N-acetyl-L-cysteine (0.4 mmole) was dissolved in aq. 50% (v/v) pyridine (5 ml) at RT and then heated to 45°C. The pH of solution was adjusted to 9 with 1 M NaOH. To this solution was added, dropwise, BITC and PEITC (0.8 mmole). The reaction was kept at 45°C for 1 hr. After cooled on ice, the reaction mixture was extracted with ice-cold benzene 5 × 5 ml, and the extracts were discarded. On adjusting the remaining aq. solution to pH 1 with 1 M HCl, the white and sticky product was precipitated. The suspension was extracted with CH₂Cl₂ 5 × 5 ml. The product was obtained from CH₂Cl₂ layer after dried in vacuo, and was further purified by column chromatography with silica gel (60 – 200 mesh) eluted by gradient CH₂Cl₂/MeOH and preparative TLC with CH₂Cl₂/MeOH (85:15). Characterization was by NMR and LC/MS.

LC/MS/MS Assay for PEITC

The HPLC assay lacked the sensitivity needed to determine PEITC concentrations in plasma and urine samples. We have spent considerable time trying to develop LC/MS/MS assays for PEITC, BITC and NITC. Possible reasons for the lack of success of the LC/MS assay for PEITC and BITC include: 1) *Low molecular weight*. PEITC and BITC have molecular weights of 163.2 and 149.22, respectively. They fall into the noise range of LC/MS. 2) *High volatility*. PEITC is volatile and easily to evaporate under vaporization of the mobile phase. Therefore, only trace amounts enter the mass spectrometer. 3) *Low polarity*. PEITC is not easily ionized by electrospray ionization (ESI) in LC/MS due to its low polarity. 4) *Low aqueous stability*. Therefore, we investigated a GC/MS assay for PEITC using a Hewlett Packard 5972 Mass selective detector with a 5890 Series II gas chromatograph, data station and 7673 auto sampler. The separation used a 30m × .25mm capillary column HP-5 (5% phenyl methyl silicone), injection temperature of 250 °C, column temperature of 170 °C, and helium flow rate of 1ml/min. The mass spectrometric conditions were: electron ionization (EI) source and detector temperature 200 °C. Selected ion monitoring of fragments of m/z 163, 105 and 91 is used for analysis of PEITC. These procedure again did not prove to be useful, due to the high volatility of PEITC during the extraction procedure, where greater than 70% loss of PEITC was found.

A different LC/MS/MS approach was then investigated, which involved the derivatization of PEITC. This assay has been published (Ji and Morris, Anal Chem 323:39-47, 2003) and the manuscript is present in Appendix. β-phenylethyl-1,1,2,2-(²H₄) amine was used to synthesize β-phenylethyl-1,1,2,2-(²H₄) isothiocyanate, the internal standard for PEITC. The synthesis is shown below:



Abstract. Phenethyl isothiocyanate (PEITC) is a dietary compound present in cruciferous vegetables that has cancer preventive properties. Our objective was to develop and validate a novel liquid chromatography-tandem mass spectrometry (LC/MS/MS) procedure to analyze PEITC concentrations in human plasma and urine. Following hexane extraction, ammonia was added to samples to derivatize PEITC to phenethylthiourea. Chromatographic separation was achieved on a C₁₈ column with acetonitrile/5 mM formic acid (60:40, v/v) as the mobile phase followed by tandem mass spectrometry detection in multiple reaction monitoring mode. Deuterium-labeled PEITC was used as the internal standard. The detection limit was 2 nM and calibration curves were linear from 7.8 to 2000 nM. The intra- and inter-day coefficients of variation were less than 5% and 10% respectively. The intra- and inter-day accuracy ranged from 101.0 to 104.2% and 102.8 to 118.6%, respectively. The recovery from spiked human plasma and urine ranged from 100.3 to 113.5% and 98.3 to 103.9%, respectively. The assay was used to measure PEITC in plasma and urine samples obtained from subjects after consumption of 100 g of watercress. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentration of PEITC.

Analysis of PEITC-NAC (mercapturic acid conjugate of PEITC)

This is the first report on mass spectra of N-acetyl-S-(N-phenylethylthiocarbamoyl)-L-cysteine, the mercapturic acid metabolite of phenethyl isothiocyanates (PEITC) in human and rat samples. The mass spectra were performed on a Perkin-Elmer SCIEX API 3000 triple-quadrupole mass spectrometer (PE SCIEX, Foster City, CA) by infusing the sample at the rate of 10 µl/min in combination with a flow rate of 0.1 ml/min in a mobile phase of acetonitrile:water 95/5. The instrument was equipped with a turboion-spray source operated in the negative ionization mode. Nitrogen was used as the nebulizer, drying and curtain gases. The turboion-spray probe was maintained at 400°C. The stainless-steel capillary needle was charged at -5.0 kV. Full scan spectra were acquired in single-quadrupole mode (Q1-scan) with a scan range of 150 - 1,000 amu. The declustering potential was set -20 V. Product ion spectra of the selected molecular ions [M - H]⁻ at m/z 325 were acquired by MS/MS using varying collision energies (CE = -5 ~ -35 V). The spectral data were analyzed via Analyst Software Version 1.1.

The molecular ion of N-acetyl-S-(N-phenylethylthiocarbamoyl)-L-ysteine was at m/z 325.1 determined as [M - H]⁻ in full scan spectra from 150 to 1,000 amu. The molecular ion can be further confirmed by its dimer at m/z 673.4 {[M - H] × 2 + Na}⁻ and 713.3 {[M - H] × 2 + Na + K + H}⁻, respectively. Under the current condition of Q1 scan, the molecular ion [M - H]⁻ was stable enough to be detected in negative ionization mode. MS/MS spectra from the selected molecular ion [M - H]⁻ at m/z 325 demonstrated the product ions at m/z 196, 162 and 84. The

data indicated that N-acetyl-S-(N-phenylethylthiocarbamoyl)-L-cysteine could be qualified and quantified by LC/MS/MS in metabolite studies.

b) NITC

This assay has been published (Hu and Morris, J Chromatogr B 788:17-28,2003) and is present in the Appendix.

Abstract. A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for determination of α -naphthylisothiocyanate (1-NITC) and two metabolites α -naphthylisothiocyanate (1-NA) and α -naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Partisphere C-18 5 μ m column, a mobile phase acetonitrile/water (ACN:H₂O 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. 1-NITC and 1-NA were found in rat plasma and urine samples, respectively, with retention time 5.9 and 2.2 min by elution of. The lower limits of quantitation (LLQ) in rat plasma, urine, and ACN were 10, 30, and 10 ng/ml for 1-NITC; 30, 100, and 30 ng/ml for 1-NA; and 30 ng/ml in ACN for 1-NIC. The standard curve was linear over a concentration range of 10-5000 ng/ml for 1-NITC in plasma and 100-50000 ng/ml for 1-NA in urine ($r > 0.999$). At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the within-day and between-day accuracy were 95-106% and 97-103% for 1-NITC in plasma, respectively. The values for within- and between-day precision were 97-100% and 93-97%, respectively. For 1-NA in urine, the within- and between-day accuracy and precision values were 96-106% and 97-99%, respectively. ACN extraction of plasma and urine samples resulted in recoveries of 1-NITC between 93 and 97%, and of 1-NA between 95 and 110%. The stabilities of 1-NITC, 1-NA, and 1-NIC for time course were studied in rat plasma, urine, ACN extracts of plasma and urine, ACN, and universal buffer (citrate-phosphate-borate-HCl, pH 2-12) at room temperature (RT), 4, -20, and -80°C over 96 h. The stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at -20 and -80°C in plasma, urine, and ACN extracts of plasma and urine, but degraded at RT and 4°C. In universal buffer at RT, 1-NITC degraded rapidly at pH values ranging from 2 to 12. 1-NA was stable in all tested matrix at all temperatures. 1-NIC was unstable in plasma, urine, and ACN extracts of plasma and urine, but stable in ACN. The degradation product of 1-NITC and 1-NIC in universal buffer was confirmed to be 1-NA. The validated HPLC assay was used in a pharmacokinetic study of 1-NITC following intravenous (i.v.) bolus administration of 25 mg/kg dose to a female Sprague-Dawley rat. 1-NITC and 1-NA were detected and quantified in rat plasma and urine. Based on noncompartmental analysis, values for clearance (CL = 2.07 l/kg/h), volume of distribution (V = 14.3 l/kg), and half life ($t_{1/2} = 4.76$ h) were determined for 1-NITC.

c) BITC

HPLC Assay

A Waters 1525 Binary Pump and a Waters 2487 Dual λ Absorbance Detector were used. Sample injection was achieved with a Waters 717plus Autosampler, equipped with a Heater/Cooler (25 °C). The column was PartiSphere WVS C18 (5 μ m), 4.6 X 125 mm I.D. from Whatman. The column temperature (25 °C) was controlled by a Waters 1500 Column Heater. The mobile phase consisted of a mixture of 60% methanol and 40% water eluting at a flow rate of 1.0 ml/min. BITC eluted with a retention time of 7 min. UV detection was optimized to 245 nm.

Stability of BITC. The stability of BITC was determined at various pH values (3, 5, 7.4, 8.4, 9.4, and 10.4) and over time (120 hours). A universal buffer of citrate-phosphate-borate/HCl A universal buffer of citrate-phosphate-borate/HCl (Teorell & Stenhagen) was used in this study. The buffer solutions, with pH of 3.0, 5.0, 7.4, 8.4, 9.4 and 10.4, were spiked with a 0.1mM BITC (in acetonitrile). All samples were left at room temperature and injected at various times throughout a 120-hour period. Injection volume was 50 μ l. The stability of BITC is summarized in Table 1. BITC was found to degrade in buffer solutions and the degradation rate followed first order kinetics (Fig. 1). The results showed that BITC is more stable in an acidic environment than in an alkaline one, and is most stable between pH 5 and 7.

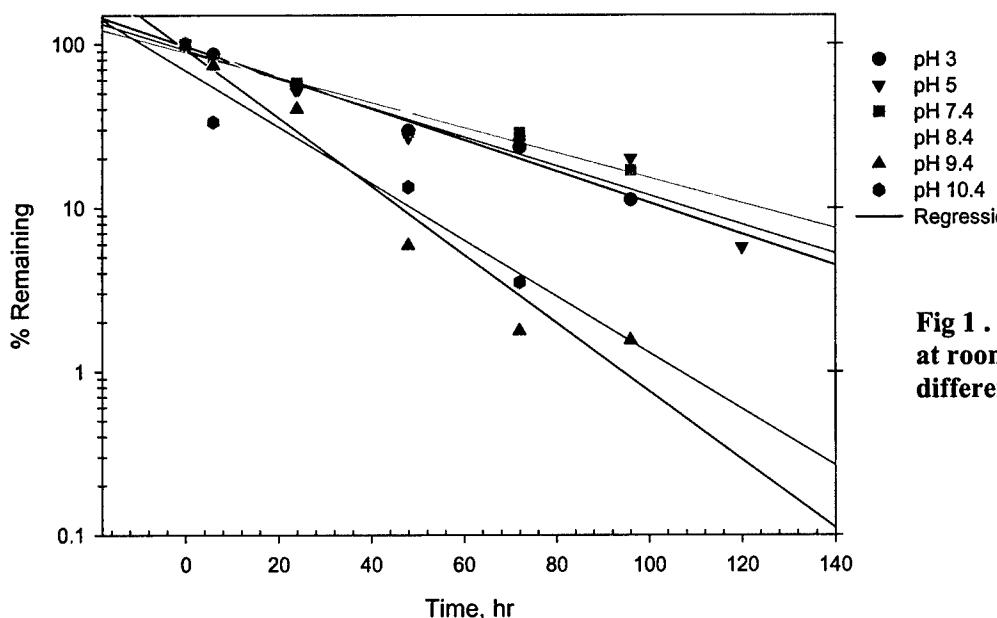


Fig 1 . Degradation of BITC at room temperature at different pH values.

Table 1. Parameters for the first order degradation of BITC in buffer solutions at various pH values.

| pH | 3 | 5 | 7.4 | 8.4 | 9.4 | 10.4 |
|-----------------------------------|-------|-------|-------|-------|-------|-------|
| K value (hours ⁻¹) | 0.022 | 0.020 | 0.018 | 0.030 | 0.048 | 0.173 |
| Half-life (hours) | 31.5 | 34.0 | 39.4 | 22.9 | 14.4 | 14.0 |

Task 4. Perform in vivo pharmacokinetic studies in rats.

Overview: Pharmacokinetic studies for NITC and PEITC have been completed. Our progress has been slow in this area due to the time that was necessary to develop assay methods with the specificity and sensitivity needed to analyze plasma concentrations following relevant (low) doses. Two manuscripts are presently being finalized for journal submission. The NITC manuscript (Hu and Morris) is included in the Appendix.

a) Pharmacokinetics of NITC in rats.

Abstract. Many naturally occurring and synthetic isothiocyanates (ITCs) have been reported to inhibit chemical carcinogenesis in animal models. More recently, we found that α -naphthyl isothiocyanate (1-NITC), a synthetic ITC, significantly increased the accumulation of the anticancer drug daunomycin in P-glycoprotein (P-gp) and multidrug resistance associated protein 1 (MRP1) overexpressed cells, indicating the potential application of 1-NITC as a chemosensitizing agent for cancer chemotherapy [Hu and Morris, *J Pharm Sci* 93: 1901-1911, 2004]. The objective of this study was to explore the pharmacokinetic characteristics of 1-NITC in rats. A single dose of 10, 25, 50 or 75 mg/kg of 1-NITC was administered intravenously or orally to female Sprague-Dawley rats ($n = 4$ for each group). Dose-normalized concentration-time profiles were not superimposable following intravenous or oral dosing groups, indicating that the disposition of 1-NITC in rats was nonlinear. As doses increased from 10 to 75 mg/kg following intravenous administration, the total clearance (CL) decreased from 2.2 ± 0.9 to 0.8 ± 0.3 L/h; oral availability averaged 0.46 for oral doses of 10-75 mg/kg. A nonlinear two-compartment open model with capacity-limited absorption and capacity-limited elimination from the central compartment best fit the data, based on goodness-of-fit criteria. The mechanism underlying the nonlinear elimination of 1-NITC in rats is most likely due to the capacity-limited metabolism of 1-NITC mediated by cytochrome P450 and glutathione S-transferase enzymes. We propose that the nonlinear absorption of 1-NITC is not due to saturable transport in the intestine, but reflects a rate-limiting step, the active transport of the labile glutathione conjugate of 1-NITC into bile, followed by its rapid hydrolysis and the rapid absorption of the lipophilic 1-NITC. This study represents the first report of the pharmacokinetics of 1-NITC; this information will be useful for optimizing dosing regimens of 1-NITC, as a multidrug resistance reversal agent.

b) Pharmacokinetics and Bioavailability of PEITC in rats.

Purpose: Phenethyl isothiocyanate (PEITC), a dietary compound in cruciferous vegetables, has chemopreventive properties and is being investigated in Phase I clinical studies. The pharmacokinetics of PEITC are largely unknown. The objective of this study was to examine the pharmacokinetics of PEITC in rats following oral and intravenous administration.

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 $\mu\text{mol}/\text{kg}$ i.v. or 10, 100 or 400 $\mu\text{mol}/\text{kg}$ orally. PEITC was prepared in 15% hydroxy-propyl- β -cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{\max} (t_{\max}) increased, maximal plasma concentrations (C_{\max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, Cl was decreased while V was increased. The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

Table 2. Pharmacokinetic parameters of PEITC in rats

| Dose ($\mu\text{mol/kg}$) | 2 | 10 | 100 | 400 |
|--------------------------------|-----------------|-----------------|-------------------|----------------------------------|
| $t_{1/2}$ (h) | 3.52 ± 0.35 | 6.92 ± 3.73 | $9.19 \pm 0.83^*$ | $13.1 \pm 2.0^{***\Delta\Delta}$ |
| Cl (L/h/kg) | 0.70 ± 0.17 | 0.68 ± 0.29 | 0.36 ± 0.18 | 0.50 ± 0.04 |
| V (L/kg) | 3.52 ± 0.63 | 7.82 ± 5.66 | 4.94 ± 2.84 | 9.46 ± 2.06 |
| t_{max} (h) | | 0.4 ± 0.1 | 2.0 ± 1.0 | $2.3 \pm 1.2^\Delta$ |
| C_{max} (μM) | | 9.2 ± 0.6 | 42.1 ± 11.4 | 48.0 ± 5.9 |
| F (%) | | 115 | 92.4 | 63.8 |

Abbreviations for the parameters: $t_{1/2}$, elimination half-life; Cl, clearance; V, volume of distribution; C_{max} , maximal plasma concentration; t_{max} , time to reach C_{max} ; F, bioavailability. The parameters were calculated by non-compartmental analysis. Statistics were conducted by ANOVA followed by Bonferroni's test, $n = 3$ or 4; * $P < 0.05$ and *** $P < 0.001$ compared to 2 $\mu\text{mol/kg}$ group; $^\Delta P < 0.05$ and $^{\Delta\Delta}P < 0.01$ compared to 10 $\mu\text{mol/kg}$ group.

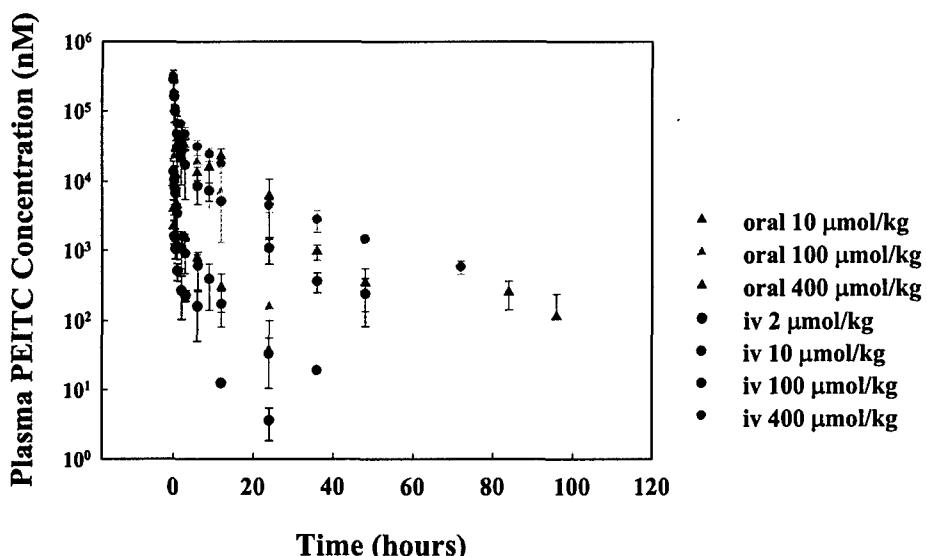


Fig. 2. The plasma concentration profile of PEITC in rats after iv and oral administration.

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses.

Task 5. Set up HPLC assay for doxorubicin and its metabolites.

Overview:

A HPLC assay using fluorescence detection was set up in the laboratory for the analysis of doxorubicin. The analysis of DOX by high-performance liquid chromatography (HPLC) methods with fluorescence detection has been well documented in a variety of biological samples. An HPLC assay was set up and the lower limit of quantitation (LLQ) and linearity for the standard curve were determined in the present study.

HPLC Assay for Doxorubicin

The Waters HPLC system (Milford, MA) consisted of a 1525 binary pump, a 717plus autosampler, a Model 5HC column oven, a 1525 fluorescence detector, and a 2487 UV detector. The column and autosampler temperatures were at room temperature. The reversed-phased chromatography was performed with a stain-steel less Whatmann (Clifton, NJ) Partisphere C-18 5 μ m column 125 \times 4.6 mm protected by a μ Bondapak-CN precolumn insert, and eluted isocratically with a mobile phase consisted of H_2O :acetonitrile (30:70, v/v). The flow rate was set at 1.0 ml/ml and the injection volume was 10 μ l. The eluent was monitored fluorimetrically at an excitation wavelength of 480 nm and an emission wavelength of 560 nm. The detection and integration of chromatographic peaks was performed by the Breeze data analysis system.

The LLQ of DOX by HPLC with fluorescence detection was identified as 10 ng (1 μ g/ml with 10 μ l injection) since the height of sample peak is 10-fold greater than the noise level. The retention time (R_t) for DOX was 5.44 min. The other peak with a R_t around 7 min was identified as some interference(s) in the solvent ACN. (Fig. 3) Our data are consistent with previously reported LLQ data, which range from 2.5 to 20 ng.

Based on the result of LLQ, the linearity of standard curve was tested from 1.0 to 10.0 μ g/ml with the same injection (i.e. 10 μ l). The correlation coefficient r was greater than 0.999.

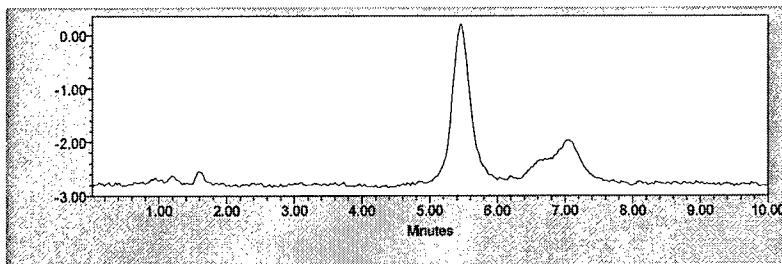


Fig. 3. HPLC assay of doxorubicin (10 μ l of a 5 μ g/ml solution was injected)

LC/MS/MS Assay for Doxorubicin.

We set up an LC/MS/MS assay for doxorubicin (DOX) that was developed in our department, by the consultant for this grant, Dr. Straubinger, and used DOX and liposome encapsulated DOX. Samples (10 μ L) were introduced into a Perkin Elmer - Sciex API 3000 liquid chromatography tandem mass spectrometer (LC-MS/MS) via a turbo ion-spray source in positive ion mode. Separation was achieved under isocratic conditions using a reversed phase C18 guard and an analytical column at a flow rate of 250 mL/min. An optimal mobile phase consisted of water:acetonitrile (60:40 v/v) containing 5 mM ammonium acetate (pH 3.5) provided sufficient separation from the void front and resulted in analysis times of 4 minutes. Assay performance (i.e., selectivity, sensitivity, linearity, and accuracy) was determined by injection of standards and quality control solutions prepared in blank plasma or tissues of interest. Plasma, liver, spleen, heart, lung, brain, and brain-tumors were harvested from Fisher 344 rats bearing 9L brain tumors after weekly administration of 5.67 mg/kg of free or DOX encapsulated in long circulating liposomes (SSL-DOX). Extraction efficiencies of 80-112% were achieved reproducibly for tissues examined. Instrumental parameters were optimized for parent and product ions for DOX and metabolites. The LC-MS/MS assay was linear over the range of

therapeutically relevant plasma/tissues concentrations (0.247-1000 nM), with a lower limit of quantification of 0.247 nM and a sensitivity of ~2.8 pg achieved in brain tissue. Intra-day coefficients of variation for all tissues were less than 20%. LC-MS/MS permitted quantification of low concentrations of DOX present in small animal tissue samples.

Task 6. Evaluate the effects of NITC, BITC and PEITC on the blood and urinary concentrations of unchanged DOX and its major metabolites following the i.v. administration of both the free and liposomal forms of DOX (7.5 mg/kg) in rats *in vivo*.

These studies are on-going. We have performed studies examining the effect of another compound, biochanin A, on DOX plasma concentrations in rats, so all methodology is in place for these studies.

Task 7. Evaluate the effects of NITC, BITC and PEITC on the metabolism and biliary excretion of DOX in the perfused rat liver.

Overview.

We chose to determine the potential effect of PEITC on metabolizing enzymes by evaluating the effect of a 4-day incubation with PEITC (3 μ M) on the gene expression in human hepatocytes (In Vitro Technologies). This technique allowed us to examine a wide range of enzymes. We used the GEArray[®] Q series Human Drug Metabolism Superarrays containing 96 genes encoding for drug metabolizing enzymes. Briefly, cDNA probes were synthesized by reverse transcription using 1 μ g of the control (vehicle treatment) or treated RNA samples as the templates and labeled with α -³²P-dCTP (10 μ Ci/ μ l; 3000 Ci/mmol, Amersham Pharmacia BioTech, Piscataway, NJ). The cDNA probes are then denatured and hybridized with GEArray[®] membranes. The hybridization signal was detected with a phosphor imager (Packard Instruments, Meriden, CT) and the relative abundance of a particular transcript was normalized against the signal of β -actin. The differences between the control and treated RNA samples were evaluated by significant analysis of microarrays (SAM) and student's t-test. There were no significant differences detected. This may reflect the variability observed with the hepatocyte preparations.

Conclusions: At a concentration of 3 μ M of PEITC, we did not observe any significant changes in mRNA expression of drug metabolizing enzymes. This does not rule out direct inhibition of metabolizing enzymes.

Task 8. Set-up a murine animal model of breast cancer through the s.c. implantation of both resistant and sensitive MCF-7 cells. Methods will be set up to determine antitumor effect and toxicity.

We will begin setting up our animal model this summer with Dr. Atif Awad (Nutrition Program, University at Buffalo), who has considerable experience with xenograft murine cancer models.

Task 9. Evaluate the effect of NITC, PEITC and BITC on the efficacy and toxicity of DOX, administered in both free and liposomal forms at a dose of 6-10 mg/kg in the murine breast cancer model

These last studies examining the effect of ITCs on DOX efficacy in a mouse xenograft model have not been performed, and will be performed in the next year.

We were interested in pursuing some other studies based on our research findings from this grant, in order to gain additional information prior to performing the murine cancer model studies. These studies represented extensions of our specific aims and included examining the direct cytotoxicity of ITCs and examining the effects on other efflux transport proteins important for MDR. Both of these studies would assist in the interpretation of the proposed *in vivo* studies.

Studies were performed in three different areas, and we have written 3 papers based on this research.

Overview.

We were interested in pursuing the mechanism of interactions of ITCs with P-glycoprotein and Multidrug Resistance Protein 1 (MRP1) before performing *in vivo* studies. Secondly, with regards to the effects of ITCs in cancer prevention or treatment we needed to examine the cytotoxicity of these compounds and compare them to chemotherapeutic agents. These studies were performed in human breast cancer MCF-7 cells and in normal mammary gland cells MCF-12A. Thirdly, we were very interested in a new MDR protein, Breast Cancer Resistance Protein (BCRP, ABCG2) and wanted to determine effects of ITC on this ABC protein before going on to evaluate *in vivo* effects in MDR. The results of these studies are summarized below.

a) Mechanism of Interaction of ITCs with P-glycoprotein and MRP1.

This paper has been published (Hu and Morris, J Pharm Sci 93: 1901-1911, 2004) and is present in the Appendix. The abstract is given below.

Abstract. The objective of this investigation was to evaluate the effects of two dietary isothiocyanates (ITCs), benzyl- (BITC) and phenethyl isothiocyanate (PEITC), and one synthetic ITC, α -naphthyl isothiocyanate (1-NITC), on the P-glycoprotein (P-gp)- and multidrug-resistance protein 1 (MRP1)-mediated efflux of daunomycin (DNM), determine whether PEITC is a substrate of P-gp and/or MRP1, and elucidate the mechanism(s) involved in the inhibition of transport. BITC, PEITC and 1-NITC significantly increased the 2-h accumulation of DNM in MCF-7/ADR (P-gp overexpression), PANC-1 (MRP1 overexpression), and human colon adenocarcinoma Caco-2 cells (except for 1-NITC). The accumulation of ^{14}C -PEITC was not changed in Caco-2, human breast cancer MDA435/LCC6 and MDA435/LCC6MDR1 (P-gp overexpression) cells in the absence and presence of the P-gp inhibitor verapamil, but significantly increased with the MRP inhibitor MK571 in PANC-1 cells. The isocyanate and amine metabolites had no effect on DNM accumulation in any cell line. After 2- and 24-h ITC treatments, cellular concentrations of glutathione (GSH) in PANC-1 and Caco-2 cells were depleted by BITC and PEITC, but not by 1-NITC; glutathione-S-transferase activity exhibited small changes. Our results suggest that 1) BITC, PEITC and 1-NITC inhibit the P-gp- and MRP1-mediated efflux of DNM; 2) PEITC and/or its conjugates do not represent P-gp substrates; 3) BITC and PEITC, but not 1-NITC, inhibit MRP1 through the depletion of intracellular GSH which acts as a co-substrate for DNM efflux via MRP1; and 4) PEITC and/or its conjugates are MRP1 substrates so binding interactions with DNM represent a second potential mechanism involved in MRP1 inhibition.

b) Cytotoxicity Studies. Studies were conducted to evaluate the direct cytotoxicity of the three ITCs, since we wanted to determine direct effects on cell growth of cancer cells, before looking at concomitant therapy with DOX. This manuscript is now in press in Exp. Biol. Med. and the journal proofs are in the Appendix. Our results are summarized below:

Abstract. Organic isothiocyanates (ITCs) (mustard oils) are dietary components, present in cruciferous vegetables. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC), β -phenethyl isothiocyanate (PEITC) and sulforaphane in the human breast cancer MCF-7 and human mammary epithelium MCF-12A cell lines, as well as in a second human epithelial cell line, human kidney HK-2 cells. The cytotoxicity of PEITC, BITC, NITC and sulforaphane, as well as the cytotoxicity of the chemotherapeutic agents daunomycin (DNM) and vinblastine (VBL), were examined in MCF-7/sensitive (wt), MCF-7/Adr (overexpresses P-glycoprotein), MCF-12A and HK-2 cells. Cell growth was determined by a sulforhodamine B assay. The IC₅₀ values for DNM and VBL in MCF-7/Adr cells were $7.12 \pm 0.42 \mu\text{M}$ and $0.106 \pm 0.004 \mu\text{M}$ (mean \pm SE) following a 48-hour exposure; IC₅₀ values for BITC, PEITC, NITC and sulforaphane were 5.95 ± 0.10 , 7.32 ± 0.25 , 77.9 ± 8.03 and $13.7 \pm 0.82 \mu\text{M}$, with similar values obtained in MCF-7/wt cells. Corresponding values for BITC, PEITC, NITC and sulforaphane in MCF-12A cells were 8.07 ± 0.29 , 7.71 ± 0.07 , 33.6 ± 1.69 , and $40.5 \pm 1.25 \mu\text{M}$, respectively. BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells, at concentrations similar to the chemotherapeutic drug DNM. Sulforaphane and NITC exhibited higher IC₅₀ values. The effect of these ITCs on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppression of the growth of preclinical tumors, and/or may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.

c) Effect of Organic Isothiocyanates on Breast Cancer Resistance Protein (ABCG2)-Mediated Transport.

Breast Cancer Resistance Protein (BCRP, ABCG2) is a member of the ATP Binding Cassette (ABC) proteins, and represents another membrane protein than may be responsible for MDR in cancer. Our studies represent the first investigation of the effect of ITCs on BCRP-mediated transport. We have submitted a manuscript on our findings showing that ITCs are potent inhibitors of BCRP. The manuscript is currently under review (returned for revisions) and is included in the Appendix.

Abstract.

Purpose. To investigate the effect of organic isothiocyanates (ITCs), dietary compounds with chemopreventive activity, on breast cancer resistance protein (BCRP)-mediated transport.

Methods. The effect of twelve ITCs on the cellular accumulation of mitoxantrone (MX) was measured in both BCRP-overexpressing and BCRP-negative human breast cancer (MCF-7) and large cell lung carcinoma (NCI-H460) cells by flow cytometric analysis. The ITCs showing activity in MX accumulation were examined for their effect on MX cytotoxicity and the intracellular accumulation of radiolabeled PEITC was measured in both BCRP-overexpressing and BCRP-negative NCI-H460 cells.

Results. ITCs significantly increased MX accumulation and reversed its cytotoxicity in resistant cells, but had a small or no effect in sensitive cells. The effects of ITCs on MX accumulation and cytotoxicity were ITC-concentration dependent. Significant increases in MX accumulation were

observed at ITC concentrations of 10 or 30 μ M and significant reversal of MX cytotoxicity was generally observed at ITC concentrations of 10 μ M. Intracellular accumulation of radiolabeled PEITC in BCRP-overexpressing cells was significantly lower than that in BCRP-negative cells, and was increased significantly by the BCRP inhibitor fumitremorgin C (FTC).

Conclusions. Certain ITCs are BCRP inhibitors and PEITC and/or its cellular metabolite(s) may represent BCRP substrates, suggesting the potential for diet-drug interactions.

Review Papers.

We have written two review papers (present in Appendix), and are currently working on an additional paper.

- a) We have published a review paper on gender differences in membrane transport to evaluate potential differences in transport by P-glycoprotein and other transport proteins in women and men. This represents an important consideration in the interindividual differences in pharmacokinetics and efficacy of anticancer drugs.
- b) We have written and submitted a review paper (accepted) on efflux transporters in drug elimination, which includes a review of P-glycoprotein, MRP transporters and breast cancer resistance protein, all potential targets of isothiocyanates.
- c) We have been invited to write a review paper on Isothiocyanates and Cancer Prevention for a book entitled Nutrition and Cancer Prevention. This is in progress.

KEY RESEARCH ACCOMPLISHMENTS

- a) Organic isothiocyanates, also known as mustard oils, are present in cruciferous vegetables in mg quantities. We reported for the first time that three organic isothiocyanates (α -naphthyl isothiocyanate (NITC), phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC)) inhibit the P-glycoprotein-mediated efflux of daunomycin and vinblastine. We determined that PEITC is not a substrate for P-glycoprotein. Additionally, the isocyanate and amine metabolites of PEITC, BITC and NITC do not affect P-glycoprotein-mediated efflux.
- b) We reported for the first time that organic isothiocyanates can inhibit the MRP1-mediated efflux of daunomycin and vinblastine, and determined that the mechanism involved, at least in part, the depletion of cellular glutathione which represents a co-substrate for the MRP1-mediated transport of anticancer drugs.
- c) We have demonstrated for the first time that PEITC, BITC and NITC are Breast Cancer Resistance Protein (BCRP, ABCG2) inhibitors and PEITC and/or its cellular metabolite(s) may represent BCRP substrates. These studies suggest the potential for reversal of MDR due to the overexpression of BCRP in cancer cells.

Therefore, PEITC, BITC and NITC are inhibitors of three ATP-dependent cassette binding (ABC) proteins that are responsible for MDR. This indicates the potential use of ITCs as MDR reversal agents for MDR due to multiple mechanisms.

- d) There is very limited data in the literature regarding the effects of ITCs on cell growth and this information is important in the evaluation of these drugs as chemosensitizing or chemopreventive agents. We found that BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells, at concentrations similar to the chemotherapeutic drug DNM. Sulforaphane and NITC exhibited higher IC₅₀ values. The effect of these ITCs on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppression of the growth of preclinical tumors, and/or may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.
- e) Reversed phase HPLC assays for NITC and BITC were developed and specificity and sensitivity determined. The assay for NITC was used to evaluate NITC and metabolite concentrations in rat plasma and urine samples.
- f) α -naphthylisothiocyanate (NITC) demonstrated dose-dependent clearance following intravenous administration; oral availability averaged 0.46 for oral doses of 10-75 mg/kg. A nonlinear two-compartment open model with capacity-limited absorption and capacity-limited elimination from the central compartment best fit the data. This study represents the first determination of the pharmacokinetics of α -naphthylisothiocyanate.
- g) A LC/MS/MS assay for PEITC was developed. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentrations of PEITC.
- h) The pharmacokinetics of PEITC were determined in rats. We have shown for the first time that the clearance of PEITC is dose dependent, and that following oral administration of PEITC, the bioavailability is high. This provides support for using PEITC as a reversal agent since oral doses are well absorbed.

REPORTABLE OUTCOMES

Abstracts (in Appendix):

1. Effect of dietary organic isothiocyanates on p-glycoprotein and MRP-mediated transport. E. Tseng, A. Kamath, W. Shi and M. E. Morris. Poster Presentation. American Association of Pharmaceutical Scientists, November 2-6, 2000. Published in Pharm. Res. (Suppl), 2000.
2. HPLC Assay for Phenethyl Isothiocyanate. K. Hewitt, I. M. Darling and M.E. Morris. West Virginia Undergraduate Pharmacy Research Symposium, October 2000.
3. Cytotoxicity of Dietary Organic Isothiocyanates in Human Breast Cancer Cells. E. Tseng and M. E. Morris. Poster presentation to the American Association of Pharmaceutical Scientists Annual Meeting, October 2001. Published in Pharmaceutical Research Suppl, 2001.
4. Effect of organic isothiocyanates on the p-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, E. Tseng, A. Kamath, and M.E. Morris. Era of Hope 2002 Meeting.

5. Determination of α -naphthylisothiocyanate and its metabolite α -naphthylamine in rat plasma and urine by high-performance liquid chromatographic assay. K. Hu and M. E. Morris, American Association of Pharmaceutical Scientists Annual Meeting, November 2002.
6. Gene Expression in Human Breast Cancer Cells Following Treatment with Phenethyl Isothiocyanate, Paul Saunders, Young Jin Moon, Daniel A. Brazeau and Marilyn E. Morris, American Association of Pharmaceutical Scientists Annual Meeting, November 2002.
7. Effects of benzyl-, phenethyl- and alpha-naphthyl isothiocyanates on P-glycoprotein- and MRP1-mediated transport of daunomycin, Hu K and Morris ME. American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.
8. Pharmacokinetics of α -naphthylisothiocyanate in rats, Hu K and Morris ME. American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.
9. The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats. Ji Y and Morris ME, to be presented at the American Association of Pharmaceutical Scientists Meeting, Baltimore MD, November 2004.

Manuscripts:

Published

1. Tseng E, Kamath AV, Zhang S and Morris ME. Effect of organic isothiocyanates on the P-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, Pharm Res 19:1509-1515, 2002.
2. Morris ME, Lee H-J, Predko LM. Gender differences in the membrane transport of endogenous and exogenous compounds, Pharmacol Rev 55:229-240, 2003.
3. Hu K and Morris ME: Determination of 1-naphthylisothiocyanate (1-NITC) and metabolites 1-naphthylamine (1-NA) and 1-naphthylisocyanate (1-NIC) in rat plasma and urine by high-performance liquid chromatography, J Chromatography B 788:17-28,2003.
4. Ji Y and Morris ME: Determination of phenethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography-tandem mass spectrometry. Anal Biochem, 323:39-47, 2003.
5. Hu K and Morris ME: Effects of benzyl-, phenethyl- and α -naphthyl isothiocyanates on P-glycoprotein and MRP1-mediated transport, J Pharm Sci, 93: 1901-1911, 2004.

Submitted/ In Press

6. Zhang S and Morris ME: Efflux transporters in drug excretion. Frontiers of Biotechnology and Pharmaceuticals (book chapter), in press.
- 7.Tseng E, Scott-Ramsay EA, Morris ME. Dietary organic isothiocyanates are cytotoxic in the human breast cancer MCF-.7 and mammary epithelial 12-A cell lines, Exp Biol Med, in press.
8. Ji Y and Morris ME Effect of Organic Isothiocyanates on Breast Cancer Resistance Protein (ABCG2)-Mediated Transport, submitted.
- 9.Hu K and Morris ME. Pharmacokinetics of α -naphthyl isothiocyanate in rats, submitted.

Presentations:

1. HPLC assay fro phenethyl isothiocyanate. Hewitt K, Darling IM and Morris ME. Undergraduate Research in Pharmacy meeting, West Virginia University, October 2000.
2. Pharmacokinetics and Metabolism of Phenethylisothiocyanate in Humans Following Watercress Ingestion, Lisa M. Predko, Yan Ji, and Dr. Marilyn E. Morris, Department of Pharmaceutics, University at Buffalo, Undergraduate research presentations, April 2002. Lisa Predko, a Pharm.D. student working with me on research projects, was the recipient of the Dr. Daniel H. Murray Professional Development Award from the School of Pharmacy and Pharmaceutical Sciences and for Best Undergraduate Poster from the Department of Pharmaceutical Sciences.
3. Benzyl isothiocyanate stability testing using high pressure liquid chromatography. Lin B, Kuo Y, Morris ME. Department of Pharmaceutical Sciences, University at Buffalo, Undergraduate research presentations, 2001.
4. Effect of Organic Isothiocyanates on the P-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, Era of Hope Meeting, Department of Defense Breast Cancer Program, Orlando FL, September 2002.
5. Yan Ji presented two departmental seminars based on her Ph.D. research for this grant.

Graduate Students Participating in this Research as a part of their educational program:
The following students participated in this project as a part of their educational program:

- Elaine Tseng, B.S/M.S (degree Sept. 2001)
M.S. Research project "Effect of Isothiocyanates on the P-glycoprotein-and MRP1-mediated transport of Daunomycin and Vinblastine"
Yushin Kuo, M.S. (degree Sept, 2003) (no salary support)
M.S. Research project "HPLC Assays and Stabilities of Phenethyl and Benzyl Isothiocyanates."
Yan Ji, Ph.D. candidate (degree expected, 2005)
Wei Liang, Ph.D. candidate (left program) (no salary support)
Shuzhong Zhang, Ph.D. candidate (degree expected, 2004)
Xiaofei Zhou, Ph.D. candidate (degree expected, 2004)

Undergraduate Students Participating in this Research as a part of their educational program:

- Krishna Hewitt, B.S. Pharmaceutics (May, 2001) (no salary support)
Bo Lin, Pharm.D. (May, 2004) (no salary support)
Lisa Predko, Pharm.D. (May 2002) (no salary support)
Heather Rochette (Biomedical Sciences Major)-Research rotation (no salary support)

Postdoctoral fellow Participating in this Research:

Ke Hu

CONCLUSIONS

- One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that benzyl-, phenethyl- and 1-naphthyl isothiocyanate inhibit the P-gp-, MRP1- and BCRP-mediated efflux of cancer chemotherapeutic drugs in MDR cancer cells. These studies are the first reports of the inhibitory effects of ITCs for all three of these ABC transporters. These compounds represent a new class of MDR reversal agents. The inhibition of multiple efflux transporters involved in MDR indicate that ITCs may be useful in MDR due to multiple causes.
- BITC and PEITC inhibit the growth of human breast cancer cells as well as human mammary epithelium cells, at concentrations similar to the chemotherapeutic drug DNM. The effect of these ITCs on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppression of the growth of preclinical tumors, and/or may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.
- HPLC assays were developed to determine PEITC, BITC and NITC concentrations in biological fluids. A novel LC/MS/MS method for PEITC was developed with the specificity and sensitivity to measure PEITC in biological fluids.
- The pharmacokinetics of PEITC and NITC were evaluated for the first time, to assess the clearance using multiple doses, mechanism of elimination and bioavailability. This information will be essential in planning in vivo studies to evaluate MDR reversal.

Overview and Recommendations for Future Work:

The progress on this grant was slow due to the time required to develop the necessary assays to determine the low concentrations of isothiocyanates and the time necessary to synthesize metabolites and standards. The development of an assay for PEITC was a time-consuming undertaking; however, we were successful in developing a novel assay which will facilitate research on isothiocyanates. My graduate student Yan Ji will continue on this research project as her Ph.D. dissertation research, hopefully to complete all specific aims.

Additionally, we chose to expand the work for several specific aims, to evaluate the inhibitory mechanisms of action of ITCs on P-glycoprotein, and to address the possibility that ITCs may inhibit other ABC efflux proteins important in MDR. This latter work proved that ITCs can inhibit efflux by MDR1 and BCRP: this makes our findings all the more valuable since now we know ITCs can potentially reverse MDR by multiple mechanisms. We have published the first data in the literature on effects of ITCs on MDR1 and BCRP.

This grant has allowed us to initiate research on this important class of dietary components. We are interested in pursuing the role of isothiocyanates as MDR reversal agents against BCRP, since the most potent effects were observed with this transport protein. Additionally, we are

interested in the chemopreventive effects of ITCs, and were able to obtain a Department of Defense Concept grant to further study PEITC.

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APPENDIX A - Abstracts

1. Effect of dietary organic isothiocyanates on p-glycoprotein and MRP-mediated transport. E. Tseng, A. Kamath, W. Shi and M. E. Morris. Poster Presentation. American Association of Pharmaceutical Scientists, November 2-6, 2000. Published in Pharm. Res. (Suppl), 2000.
2. HPLC Assay for Phenethyl Isothiocyanate. K. Hewitt, I. M. Darling and M.E. Morris. West Virginia Undergraduate Pharmacy Research Symposium, October 2000.
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Appendix 1

EFFECT OF DIETARY ORGANIC ISOTHIOCYANATES ON P-GLYCOPROTEIN- AND MRP-MEDIATED TRANSPORT

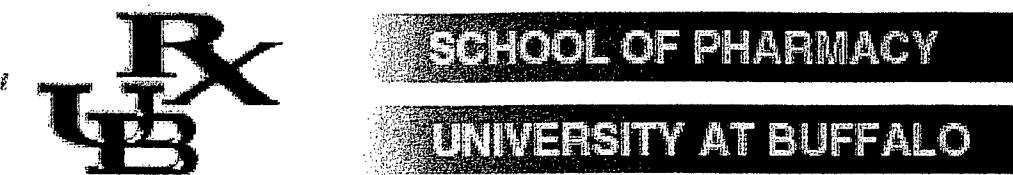
Elaine Tseng*, Amrita Kamath, Wei Shi and Marilyn E. Morris

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Purpose. Organic isothiocyanates (ITCs) (mustard oils) are present in cruciferous vegetables and known to have chemopreventative properties. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and Multidrug Resistance-Associated Protein (MRP)-mediated transport in multidrug resistant (MDR) human cancer cell lines (MCF-7 and PANC-1). **Methods.** The direct effect of organic isothiocyanates on the 2-hour cellular accumulation of daunomycin (DNM), or vinblastine (VBL), substrates for both P-gp and MRP, were measured in MCF-7 cells which overexpress P-gp and PANC-1 cells which overexpress MRP. The effect of preincubation of these substrates on DNM accumulation in resistant MCF-7 cells was also determined. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-, phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, phenylbutyl- isothiocyanate, sulforaphen, erucin, erysolin. The effect of NITC on the cytotoxicity of DNM in sensitive and resistant MCF-7 cells was also determined. **Results.** Three organic isothiocyanates (NITC, PEITC, BITC) were found to have significant inhibitory effects on the cellular accumulation of DNM and VBL in resistant MCF-7 and PANC-1 cells. Preincubation studies in MCF-7 cells demonstrated that the inhibitory effects of NITC, PEITC and BITC on DNM efflux persisted following preincubation and removal of the ITCs prior to exposure to DNM. In cell growth studies, NITC decreased the IC₅₀ value of DNM seven-fold ($0.66 \pm 0.24 \mu\text{g/ml}$ vs. $4.60 \pm 1.79 \mu\text{g/ml}$ in control, $p < 0.01$, $n=6$) in MCF-7 resistant cells with no change in the IC₅₀ in sensitive cells. **Conclusions.** One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP-mediated efflux of DNM and VBL in MDR cancer cells. Support provided by a NY Dept. of Health EMPIRE grant and grants from the Susan G. Komen Foundation and US Army MRMC (IDEA grant).

Poster Presentation. American Association of Pharmaceutical Scientists, November 2-6, 2000.
Published in Pharm. Res. (Suppl), 2000.

Appendix 2



HPLC Assay of Phenethyl Isothiocyanate

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Introduction. Phenethyl isothiocyanate (PEITC) is a naturally occurring compound present in mg quantities in cruciferous vegetables such as broccoli, cabbage and watercress (12 mg/2 oz of watercress). PEITC is of particular interest since it has been shown in animal studies to have chemopreventive properties, and is being evaluated for the chemoprevention of lung cancer. Our laboratory has also found that PEITC is an inhibitor of p-glycoprotein. However, little is known about the pharmacokinetics and metabolism of PEITC.

Objective. The objective of this investigation was to develop an HPLC assay for PEITC that could be used for serum and urine samples.

Methods. A Waters HPLC system with a Waters Model 510 pump, Model 712 automatic injector, and a Model 490E programmable multiwave detector with a 10 μ m Whatman column were used. UV absorption of PEITC was evaluated using a scanning spectrophotometer and a wavelength of 245 nm chosen for detection in the HPLC assay. The mobile phase consisted of methanol (60%) and water (40%). At a flow rate of 1 ml/min, a PEITC peak was observed at 10 min. One rat was injected with PEITC at an intravenous dose of 1 mmol/kg and urine and blood were collected.

Results. Standard curves of PEITC in serum were obtained using concentrations between 0.005 and 0.2 mM. We were able to detect PEITC in serum and urine: the serum concentration was 0.036 mM and the urine concentration was 0.83 mM. Using a similar HPLC system, we could detect other isothiocyanates, including naphthyl isothiocyanate.

Conclusions. We developed an HPLC assay for PEITC and were able to quantitate PEITC in both urine and serum samples after administration of PEITC to a rat. Further research is in progress to investigate the stability of PEITC in biological fluids and to characterize its metabolism.

Supported by grants from the Susan G. Komen Foundation and the Dept. of Defense (US Army IDEA award)

Appendix 3

Cytotoxicity of Dietary Organic Isothiocyanates in Human Breast Cancer Cells

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Purpose. Organic isothiocyanates (ITCs) (mustard oils) are present in cruciferous vegetables and known to have cancer chemopreventative properties due to their inhibition of carcinogen metabolic activation. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC) and β -phenylethyl isothiocyanate (PEITC) in a multidrug-resistant human breast cancer cell line, MCF-7/ADR. **Methods.** The cytotoxicity of PEITC, BITC, NITC, daunomycin (DNM) and vinblastine (VBL) were evaluated following the same exposure times at concentrations varying from 0.1 to 50 μ M. The compounds were added to the cultured cells in 96-well plates for an exposure period of 48 hours and cell growth was determined by a sulforhodamine B assay. DNM and VBL were used to standardize the conditions of the assay, and to obtain IC₅₀ values for comparative purposes. **Results.** Based on preliminary studies with DNM, VBL and PEITC, we found that IC₅₀'s decreased with increasing exposure times, and chose exposure times of 48 hours for our studies. The IC₅₀ values for DNM and VBL were $1.59 \pm 0.093 \mu\text{M}$ (mean \pm SE) and $0.106 \pm 0.004 \mu\text{M}$, respectively. PEITC and BITC inhibited cell growth with IC₅₀'s of $6.62 \mu\text{M} \pm 0.185 \mu\text{M}$ and $5.90 \pm 0.129 \mu\text{M}$. NITC demonstrated no cytotoxicity at concentrations of 10 μM or less. **Conclusions.** BITC and PEITC, which are present in cruciferous vegetables in mg quantities, have a cytotoxic effect comparable to the anticancer drugs, DNM and VBL, in a multidrug resistant human breast cancer cell line. NITC exhibited no cytotoxicity at similar concentrations.

Support provided by grants from NY Dept. of Health, the Susan G. Komen Foundation and the US Army MRMC (IDEA grant).

Poster presentation to the American Association of Pharmaceutical Scientists Annual Meeting, October 2001.

Published Pharmaceutical Research 18:Suppl, 2001.

**EFFECT OF ORGANIC ISOTHIOCYANATES ON
THE P-GLYCOPROTEIN AND MRP1-MEDIATED
TRANSPORT OF DAUNOMYCIN AND
VINBLASTINE**

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Purpose. Organic isothiocyanates (ITCs) (mustard oils) are non-nutrient components present in the diet, especially in cruciferous vegetables. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and Multidrug Resistance-Associated Protein (MRP1)-mediated transport in multidrug resistant (MDR) human cancer cell lines.

Methods. The direct effect of organic isothiocyanates on the 2-hour cellular accumulation of daunomycin (DNM) and vinblastine (VBL), substrates for both P-gp and MRP1, were measured in sensitive and resistant MCF-7 cells and in PANC-1 cells. Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp while PANC-1 cells overexpress MRP1. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-, phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, phenylbutyl- isothiocyanate, sulforaphane, erucin and erysolin.

Results. NITC significantly increased the accumulation of DNM and VBL in both resistant cell lines, but had no effect on DNM accumulation in sensitive MCF-7 cells. VBL accumulation in resistant MCF-7 cells was increased 40-fold by NITC, while that in PANC-1 cells was increased 5.5-fold. Significant effects on the accumulation of DNM and VBL in resistant MCF-7 cells were also observed with BITC, while PEITC, erysolin, phenylhexyl-ITC and phenylbutyl-ITC increased the accumulation of DNM and/or VBL in PANC-1 cells. Overall, the inhibitory activities of these compounds in MCF-7 cells and PANC-1 cells were significantly correlated ($r^2= 0.77$ and 0.86 for DNM and VBL, respectively). Significant effects on accumulation were generally observed with the ITCs at 50 mM concentrations, but not at 10 mM concentrations.

Conclusions. One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP1-mediated efflux of DNM and VBL in MDR cancer cells, and suggest the potential for diet-drug interactions.

Era of Hope Meeting, Sept 2002

Appendix 5

Abstract for 2002 APPS Annual Meeting

Determination of α -naphthylisothiocyanate and its metabolite α -naphthylamine in rat plasma and urine by high-performance liquid chromatographic assay. Ke Hu* and Marilyn E. Morris. Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA

Abstract

Purpose: To develop a high-performance liquid chromatographic (HPLC) assay for determination of α -naphthylisothiocyanate (1-NITC), a potential P-glycoprotein (P-gp) modulator, and its metabolite α -naphthylisothiocyanate (1-NA) in rat plasma and urine. **Methods:** The chromatographic analysis was carried out using a Partisphere C-18 5 μ m column (125 \times 4.6 mm) with a mobile phase of acetonitrile (ACN):H₂O (70:30, v/v) and UV detection at 305 nm. Naphthalene was used as the internal standard. Stability studies were performed at varying temperatures and pH values. Rat plasma and urine samples were analyzed for 1-NITC and 1-NA, following the i.v. administration of 1-NITC to a female Sprague-Dawley rat. **Results:** 1-NITC and 1-NA had retention times of 5.9 and 2.2 min, respectively. The lower limit of quantitation in plasma, urine, and ACN samples were 10, 30, and 10 ng/ml for 1-NITC, and 30, 100, and 30 ng/ml for 1-NA. The within-day and between-day accuracy and precision were 95-106% and 93-100% for 1-NITC in plasma with quality controls (QCs) of 10, 500 and 5,000 ng/ml. For 1-NA in urine, the within- and between-day accuracy and precision were 96-106% and 97-99% with QCs of 100, 5,000, and 50,000 ng/ml. The ACN extraction was efficient for both plasma and urine samples based on recoveries of 93-97% for 1-NITC, and of 95-110% for 1-NA. 1-NITC was stable at all tested temperatures in ACN, and at -20 and -80°C in plasma, urine, and ACN extracts of plasma and urine. 1-NA was stable in all tested matrix. The assay was used to analyze plasma and urine samples following administration of an i.v. dose of 25 mg/kg to a rat. 1-NITC and 1-NA were detected in plasma and urine, respectively. Based on noncompartmental analysis by WinNonLin 2.0, the fitted parameters clearance (CL = 2.07 l/kg/h), volume of distribution ($V = 14.3$ l/kg), and half life ($t_{1/2} = 4.76$ h) were determined for 1-NITC. **Conclusion:** A rapid and sensitive HPLC assay has been developed for determination of 1-NITC and its metabolite 1-NA in rat plasma and urine for future pharmacokinetic and pharmacodynamic studies.

Supported by grants from the Susan G. Komen Foundation and the U.S. Army Breast Cancer Research Program, Contract DAMD17-00-1-0376.

Accepted for American Assoc. Pharmaceutical Scientists Annual Meeting, Toronto ON November 2002.

Appendix 6

**Gene Expression in Human Breast Cancer Cells Following Treatment with
Phenethyl Isothiocyanate**

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Purpose. Phenethyl isothiocyanate (PEITC), a component in cruciferous vegetables, can block chemical carcinogenesis in animal models. Our objective was to determine the effect of treatment with PEITC on gene expression changes in MCF-7 human breast cancer cells, to evaluate potential mechanisms involved in its chemopreventive effects.

Methods. MCF-7 cells were treated for 48 hours with either PEITC 3 μ M or the vehicle. Total RNA was extracted from cell membranes preparations and labeled cDNA's representing the mRNA pool were reverse-transcribed directly from total RNA isolations for use in the microarray hybridizations. Two specific human GE Array Kits (Superarray Inc.) that contain 23 marker genes related to signal transduction pathways or cancer/tumor suppression, plus 2 housekeeping genes (β -actin and GAPDH) were utilized. Comparisons of treated versus control were evaluated in 4 cell replicates using t-tests and SAM (Statistical Analysis of Microarrays, Tusher et al., 2001). **Results.** Gene expression was significantly altered for cyclin-dependent kinase inhibitor 1C (p57 Kip2), nuclear factor kappa-B inhibitor (NF κ B), breast cancer 2, early onset (BRCA2) and CYP19 (aromatase) (determined by t-test and SAM). Increases in gene expression of 3 to 7-fold, which were significant by SAM but not a t-test, were found for BCL2-associated X protein (bax), cAMP-responsive element binding protein 2 (ATF-2) and cyclin-dependent kinase inhibitor 1A (p21Waf1). PEITC decreased the gene expression of NF κ B; this would be associated with apoptosis and delayed cell growth. PEITC treatment increased the gene expression of p57 Kip2, BRCA2, bax, ATF-2 and p21Waf1; these changes would be expected to produce decreased cellular proliferation, tumor suppression and/or apoptosis. CYP19 expression was increased by 2.4-fold.

Conclusions. PEITC treatment produced significant alterations in genes involved in tumor suppression, cellular proliferation and apoptosis. These alterations in gene expression may be important in the mechanism of the chemopreventive effects of PEITC. Supported by grants from the Susan G. Komen Foundation and the U.S. Army Breast Cancer Research Program, Contract DAMD17-00-1-0376.

Presented at the American Assoc. Pharmaceutical Scientists Annual Meeting, Toronto ON, November 2002.

Effects of Benzyl-, Phenethyl- and alpha-Naphthyl isothiocyanates on P-glycoprotein- and MRP1-Mediated Transport of Daunomycin. Ke Hu* and Marilyn E. Morris. *Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA*

Abstract

Purpose: To evaluate the effects of isothiocyanates (ITCs) on P-glycoprotein (P-gp)- and Multidrug Resistance Protein (MRP1)-mediated efflux of daunomycin (DNM), determine whether ITCs are substrates of P-gp and/or MRP1, and elucidate the mechanism(s) involved in the inhibition of transport.

Methods: The effects of benzyl- (BITC), phenethyl- (PEITC) and alpha-naphthyl isothiocyanates (1-NITC) on the 2-h accumulation of DNM in human breast cancer MCF-7 and MDA435/LCC6, colonic adenocarcinoma Caco-2, and pancreatic adenocarcinoma PANC-1 cells were evaluated. Verapamil (VRP, P-gp inhibitor) and MK571 (MRP1 inhibitor) were used as positive controls. ¹⁴C-PEITC was used for substrate studies in MDA435/LCC6, MDA435/LCC6MDR1, Caco-2 and PANC-1 cells in the absence and presence of VRP or MK571. Cellular concentrations of glutathione (GSH) and activities of glutathione-S-transferase (GST) were measured after 2- and 24-h drug treatment in PANC-1 and Caco-2 cells.

Results: BITC, PEITC and 1-NITC significantly increased the accumulation of DNM in MCF-7/ADR, Caco-2 (except for 1-NITC), and PANC-1 cells. The uptake of PEITC was not changed by VRP in Caco-2, MDA435/LCC6 and MDA435/LCC6MDR1, but significantly increased by MK571 in PANC-1 cells. Cellular GSH was profoundly depleted in PANC-1 and Caco-2 cells by BITC and PEITC (6-100-fold), but not by 1-NITC. GST activities were not changed with treatment.

Conclusion: ITC group, aryl rings and the length of alkyl chains play key roles in reversal activity, besides Log P. PEITC is a substrate of MRP1 rather than P-gp. It is probably that the increased accumulation of DNM by BITC and PEITC is due to the dramatic depletion of cellular GSH (co-substrate for DNM efflux) and the competitive binding of glutathione conjugates (ITC-SGs) to D-site of MRP1 with DNM. The mechanism of 1-NITC has not been known. The inactivity of 1-NITC to increase DNM uptake in Caco-2 cells is most likely due to its extensive metabolism by cytochrome P450 1A1.

Pharmacokinetics of α -Naphthylisothiocyanate in Rats. Ke Hu* and Marilyn E. Morris.

*Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,
State University of New York at Buffalo, Buffalo, NY 14260, USA*

Abstract

Purpose: To investigate pharmacokinetics of α -naphthylisothiocyanate (1-NITC) in rats.

Methods: Pharmacokinetic studies of 1-NITC were performed with four doses of 10, 25, 50 and 75 mg/kg to Sprague-Dawley female rats ($n = 4$ for each group; body weight 200-250 g) via i.v. administration. Blood samples (250 μ l each) were collected from the jugular vein at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, 24, 36 and 48 h (36 and 48 h for 50 and 75 mg/kg groups). The concentrations of 1-NITC in plasma were determined by HPLC assay with C18 column (125 \times 4.6 mm i.d., 5 μ m), a mobile phase consisting of ACN-H₂O (70:30, v/v), flow rate at 1.0 ml/min, and the detection wavelength at UV 305 nm. The data were simultaneously fitted using ADAPT II software.

Results: 1-NITC exhibited nonlinear Michaelis-Menten disposition and data were characterized with a two compartment open model. Parameters were estimated as: maximum velocity (V_{max}), 2.13 ± 0.20 mg/h/kg; Michaelis-Menten constant (K_m), 0.51 ± 0.13 mg/L; first order rate constant from central to tissue compartment (k_{12}), 1.10 ± 0.15 h⁻¹; first order rate constant from tissue to central compartment (k_{21}), 0.32 ± 0.04 h⁻¹; volume of central compartment (V_C), 3.37 ± 0.21 L/kg; volume of tissue compartment (V_T), 11.72 ± 0.85 L/kg.

Conclusion: 1-NITC demonstrated nonlinear pharmacokinetics via i.v. administration. These results will be used to support the application of 1-NITC in combination with anticancer drug doxorubicin (DOX) to reverse P-glycoprotein (P-gp)- and Multidrug Resistance Protein 1 (MRP1)-mediated multidrug-resistance (MDR).

Appendix 9

The Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats

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Purpose: Phenethyl isothiocyanate (PEITC), a dietary compound in cruciferous vegetables, has chemopreventive properties and is being investigated in Phase I clinical studies. The pharmacokinetics of PEITC are largely unknown. The objective of this study was to examine the pharmacokinetics of PEITC in rats following oral and intravenous administration.

Methods: Male Sprague-Dawley rats were administered PEITC at doses of 2, 10, 100 or 400 $\mu\text{mol}/\text{kg}$ i.v. or 10, 100 or 400 $\mu\text{mol}/\text{kg}$ orally. PEITC was prepared in 15% hydroxypropyl- β -cyclodextrin. Plasma samples were collected at 5, 15, 30 min and 1, 2, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h and analyzed by a LC/MS/MS assay. Pharmacokinetic data were analyzed by WinNonlin for non-compartmental analysis and ADAPT II for compartmental analysis.

Results: With an increase in the PEITC dose, elimination half-life ($t_{1/2}$) and time to C_{\max} (t_{\max}) increased, maximal plasma concentrations (C_{\max}) increased but not proportionally, and oral bioavailability (F) decreased. At the highest dose, Cl was decreased while V was increased. The plasma concentration profile of PEITC after i.v. administration can be well characterized by a three-compartment model with Michaelis-Menten elimination and distribution.

| Dose ($\mu\text{mol}/\text{kg}$) | 2 | 10 | 100 | 400 |
|------------------------------------|-----------------|-----------------|-----------------|-----------------|
| $t_{1/2}$ (h) | 3.52 ± 0.35 | 6.92 ± 3.73 | 9.19 ± 0.83 | 13.1 ± 2.0 |
| Cl (L/h/kg) | 0.70 ± 0.17 | 0.68 ± 0.29 | 0.36 ± 0.18 | 0.50 ± 0.04 |
| V (L/kg) | 3.52 ± 0.63 | 7.82 ± 5.66 | 4.94 ± 2.84 | 9.46 ± 2.06 |
| C_{\max} (μM) | | 9.2 ± 0.6 | 42.1 ± 11.4 | 48.0 ± 5.9 |
| t_{\max} (h) | | 0.4 ± 0.1 | 2.0 ± 1.0 | 2.3 ± 1.2 |
| F (%) | | 115 | 92.4 | 63.8 |

Conclusions: PEITC is a dietary component with high oral bioavailability and low clearance in rats. Nonlinear elimination and distribution is evident at high doses.
Support by Contract DAMD17-00-1-0376.

Submitted to American Association of Pharmaceutical Scientists Annual Meeting, Baltimore MD, October 2004.

APPENDIX B -Manuscripts

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9. Hu K and Morris ME. Pharmacokinetics of α -naphthyl isothiocyanate in rats, submitted.

Effect of Organic Isothiocyanates on the P-Glycoprotein- and MRP1-Mediated Transport of Daunomycin and Vinblastine

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Received February 11, 2002; accepted June 12, 2002

Purpose. Organic isothiocyanates (ITCs), or mustard oils, are non-nutrient components present in the diet, especially in cruciferous vegetables. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and multidrug resistance-associated Protein (MRP1)-mediated transport in multidrug resistant (MDR) human cancer cell lines.

Methods. The direct effect of ITCs on the 2-h cellular accumulation of daunomycin (DNM) and vinblastine (VBL), substrates for both P-gp and MRP1, were measured in sensitive and resistant MCF-7 cells and in PANC-1 cells. Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp whereas PANC-1 cells overexpress MRP1. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, and phenylbutyl-ITC, sulforaphane, erucin, and erysolin.

Results. NITC significantly increased the accumulation of DNM and VBL in both resistant cell lines, but had no effect on DNM accumulation in sensitive MCF-7 cells. VBL accumulation in resistant MCF-7 cells was increased 40-fold by NITC whereas that in PANC-1 cells was increased 5.5-fold. Significant effects on the accumulation of DNM and VBL in resistant MCF-7 cells were also observed with benzyl-isothiocyanate whereas PEITC, erysolin, phenylhexyl-ITC, and phenylbutyl-ITC increased the accumulation of DNM and/or VBL in PANC-1 cells. Overall, the inhibitory activities of these compounds in MCF-7 cells and PANC-1 cells were significantly correlated ($r^2 = 0.77$ and 0.86 for DNM and VBL, respectively). Significant effects on accumulation were generally observed with the ITCs at 50 μM concentrations, but not at 10 μM concentrations.

Conclusions. One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP1-mediated efflux of DNM and VBL in MDR cancer cells and suggest the potential for diet-drug interactions.

KEY WORDS: multidrug resistance; phenethylisothiocyanate; benzylisothiocyanate; naphthylisothiocyanate; cancer chemotherapy.

INTRODUCTION

What may be considered a major setback from successful cancer chemotherapy is the phenomenon of simultaneous re-

sistance to many structurally unrelated cytotoxic agents known as multidrug resistance (MDR; 1). One well-characterized mechanism is the overexpression of efflux proteins at the surface of the cell membrane, including p-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP1). Overexpression of P-gp and/or MRP1 results in the increased efflux and therefore decreased intracellular concentrations of many natural product chemotherapeutic agents. These efflux pumps may be present at the time of diagnosis and/or may be overexpressed after drug exposure.

P-glycoprotein-mediated efflux is one mechanism of MDR that has been extensively studied. The 170kD P-gp encoded by the MDR1 gene belongs to the ATP-binding cassette (ABC) superfamily of proteins (ABCB1) and functions as an ATP-dependent efflux pump responsible for the transfer of a wide variety of xenobiotics and carcinogens from cells (2). The diverse classes of antitumor drugs that are P-gp substrates include anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes. Besides being overexpressed in various tumor cells (3), P-gp is expressed endogenously in adrenal tissues, kidney, lung, liver, and colon (4). The differential expression of P-gp in normal tissues and its conservation among species suggest that the protein may have distinct physiologic roles associated with specialized cell functions. The tissue distribution of P-gp, mainly in the epithelia of excretory organs, and the ability to transport a wide range of lipophilic substrates, are compatible with the hypothesis that P-gp serves a detoxification function in the body. In cancer cells, the overexpression of P-gp decreases the intracellular concentrations of chemotherapeutic drugs and has been positively correlated with poor prognosis in cancers (2).

Overexpression of the 190-kd multidrug resistance-associated protein (MRP1) encoded by the MRP1 gene in cancer cells also results in MDR. Although first characterized in small cell lung cancer cells (5), MRP1 is present in almost all cells of the human body, as well as overexpressed in non-P-gp MDR cell lines of the lung, colon, gastric, ovary, and breast (6). MRP1 also belongs to the family of ABC membrane transporters (ABCC1), and in a similar manner as P-gp, mediates resistance to a range of structurally and functionally unrelated agents (7). However, whereas P-gp and MRP1 both transport a number of natural product chemotherapeutic agents, substrate preferences do exist. The preferred substrates for MRP1 are usually organic anions, in particular, drugs conjugated with glutathione (GSH), glucuronate, or sulfate. In fact, MRP acts as a GS-X pump, transporting drugs conjugated to GSH out of the cell (7).

The identification and characterization of these two efflux pumps in MDR has stimulated extensive research into the search for clinically useful inhibitors. Although many inhibitors including calcium channel blockers (e.g., verapamil, nifedipine), hypotensive drugs (reserpine), antibiotics (cephalosporins, gramicidin, puromycin), immunosuppressors (cyclosporinA and its derivatives), and many other lipophilic compounds have been identified and investigated, clinical trials have been largely unsuccessful as a result of dose-related toxicities that occur at the doses necessary to achieve MDR reversal (8).

The main objective of the present study was to examine the effects of dietary organic isothiocyanates (ITCs) on P-gp-

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and MRP1-mediated transport of chemotherapeutic agents in human cancer cell lines. Organic isothiocyanates (and glucosinolates, the biosynthetic precursors of ITCs in plants), also known as mustard oils, are widely distributed in edible plants, including cruciferous vegetables, with human consumption estimated at milligram quantities daily. Glucosinolate levels have been estimated to be as high as 180 mg/g of some vegetables (9). In the present investigation we examined the effects of a range of natural and synthetic ITCs on the cellular accumulation of the P-gp and MRP1 substrates, daunomycin (DNM) and vinblastine (VBL) after 2-h exposure times. Studies were performed in sensitive and resistant human breast cancer cells (MCF-7) and human pancreatic cancer cells (PANC-1). Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp whereas PANC-1 cells overexpress MRP1.

MATERIALS AND METHODS

Erysolin, phenyl ITC, β -phenylethyl ITC, α -naphthyl ITC, and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Benzyl ITC, n-hexyl ITC, and allyl ITC were obtained from Aldrich (St. Louis, MO, USA). Sulforaphane and erucin were purchased from ICN (Aurora, OH, USA), and phenylpropyl ITC and phenylbutyl ITC were purchased from LKT Laboratories (St. Paul, MN, USA). Phenylhexyl ITC was a gift from National Cancer Institute-Chemopreventive Division (Bethesda, MD, USA). Radiolabeled [3 H]-daunomycin (14.4 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA), and [3 H]-vinblastine sulfate (7.3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Cell culture reagents were supplied by GIBCO BRL (Buffalo, NY, USA), and cell culture flasks and dishes were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA). Biodegradable liquid scintillation cocktail was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Commassie blue dye reagent was obtained from Bio-Rad laboratory (Hercules, CA, USA). The MCF-7 and MCF-7/ADR cell lines were gifts from Dr. Ralph Bernacki (Roswell Park Cancer Institute). The PANC-1 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The monoclonal antibodies C219 and MRPr1 were obtained from Kamiya Biomedical Co. (Seattle, WA, USA).

Western Analysis of P-gp and MRP1

P-gp and MRP1 expression in the cells was determined by Western analysis using the antibodies C219 and MRPr1 as described previously (10). The protein molecular weight markers (Rainbow Markers, Amersham) used were myosin (200 kd), phosphorylase b (97.4 kd), and ovalbumin (46 kd). Membrane preparations from MCF-7 and PANC-1 cells were isolated using the method of Wils *et al.* (11). Protein concentrations were measured by the Bradford method (12) using a commercially available assay kit (Bio-Rad Labs) with γ -globulin as the standard. Proteins were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose filter. The filter was blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween 20 and 1% (w/v) bovine serum albumin, incubated with C219 (1 μ g/mL) or MRPr1 (1:30) antibodies in blocking buffer for 2 h at room temperature. The filters were then washed in washing buffer

(20 mM Tris base, 137 mM NaCl, 1% Tween 20, pH 7.6) and incubated with 1:1500 (v/v) anti-mouse IgG HRP secondary antibody (Amersham; for C219) or 1:1000 anti-rat IgG HRP secondary antibody (Zymed, San Francisco, CA, USA; for MRPr1), in blocking buffer for 2 h. After washing, the protein was detected using the ECL detection reagent (Amersham). Kodak 1D image analysis software was used to analyze the Western blot results.

Cell Culture

MCF-7 and MCF-7/ADR, used between passages 16–24, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (10 units/mL), and streptomycin (10 μ g/mL). Cells were incubated at 37°C supplemented with 5% CO₂/95% air. Cells were subcultured two to three times a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 75-cm² plastic culture flasks that were seeded in 35-mm² plastic culture dishes for accumulation studies. Experiments were performed 2 to 3 days after seeding.

PANC-1 cells used between passages 60–75 were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine, sodium pyruvate, pyridoxine HCl, and 10% fetal bovine serum, which was maintained in an atmosphere of 10% CO₂/90% air at 37°C. Cells were subcultured every 2 to 3 days with 0.25% trypsin-2.6 mM EDTA. For experiments, cells were seeded on 35-mm² dishes at a density of 10⁶ cells per dish and used 2 days later.

Accumulation Studies

Growth medium was removed from monolayer cells and cells were washed twice with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂·6H₂O, 10 mM HEPES, pH 7.4). One milliliter of incubation buffer containing 0.05 μ M of [3 H]-DNM or 0.05 μ M [3 H]-VBL and 100 μ M of ITC was added to the dish and incubated for 2 h. Verapamil, a P-gp and MRP1 inhibitor, was used as a positive control in all studies. Concentration-dependent studies were performed with some of the ITCs using concentrations varying from 100 to 0.1 μ M. The uptake was stopped by aspirating the incubation buffer and washing the cells three times with ice-cold stop solution (137 mM NaCl, 14 mM Tris-base, pH 7.4). One milliliter of 0.5% Triton-X-100 or 0.3 N NaOH-1%SDS was added to each dish, and aliquots were obtained after an hour. A liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co.) was used to determine the radioactivity. The protein concentration was determined by the Bradford method (12) using a commercially available assay kit (Bio-Rad Labs) with γ -globulin as the standard.

Data Analysis

Statistical significance was determined by a one-way ANOVA followed by Dunnett's post hoc test. Differences were considered to be significant when $p < 0.05$.

RESULTS

MCF-7 Cells

Western Analysis

Western blot analyses were preformed to evaluate P-gp and MRP1 expression in MCF-7/WT, MCF-7/ADR, and

PANC-1 cells. There were undetectable amounts of P-gp in the MCF-7/WT and PANC-1 cell lines but high expression in the MCF-7/ADR cell line. PANC-1 cells showed high expression of MRP1. MCF-7/ADR cells also exhibited low expression of MRP1 (Fig. 1). The results found in this experiment confirmed those in the literature (13,14).

Time Course Study

The time course of uptake of 0.05 μM ^3H -DNM in the presence and absence of 100 μM verapamil, a typical inhibitor, was examined in sensitive (MCF-7/WT) and resistant (MCF-7/ADR) cells for up to 2 h (Fig. 2). For MCF-7/ADR cells, the accumulation of DNM was significantly greater in the presence of verapamil when compared with that in the absence of verapamil. In the sensitive cell line, which lacks P-gp, accumulation of DNM in the presence or absence of verapamil was unchanged; this demonstrates that verapamil influences the efflux of DNM through the inhibition of P-gp and not through other mechanisms in this cell line. Equilibrium conditions were achieved by 2 h in both the sensitive and resistant MCF-7 cells.

DNM Accumulation

The effect of various organic ITCs on DNM accumulation was examined in MCF-7/WT cells (Fig. 3). Verapamil did not significantly increase DNM accumulation in the sensitive cells. Only phenylpropyl ITC and phenylhexyl ITC produced significant increases in DNM accumulation in these cells. In MCF-7/ADR cells, verapamil was able to significantly increase DNM accumulation by 2.5-fold compared with the control. Few ITCs were found to inhibit the efflux of DNM, with the most active compound being 1-naphthylisothiocyanate (NITC), which increased DNM accumulation by 4-fold; benzylisothiocyanante (BITC) produced an effect that was similar in magnitude to that of verapamil 100 μM . All other compounds did not significantly alter DNM accumulation. Concentration-dependent studies demonstrated significant activity for NITC at concentrations of 50 μM but not at 10 μM (results not shown).

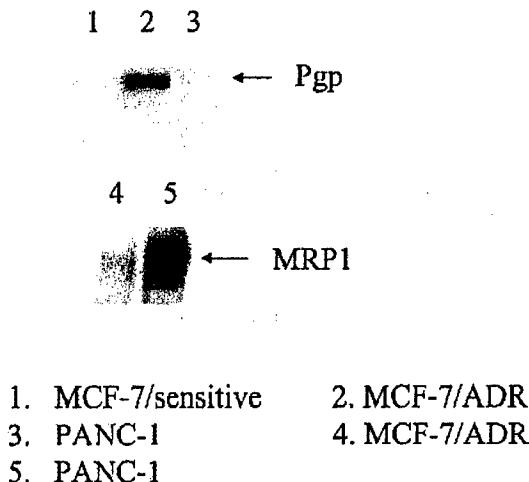


Fig. 1. Western blots of P-gp and MRP1 in MCF-7, MCF-7/ADR, and PANC-1 cells, using the antibodies C219 and MRP1, respectively (as described in the Materials and Methods section).

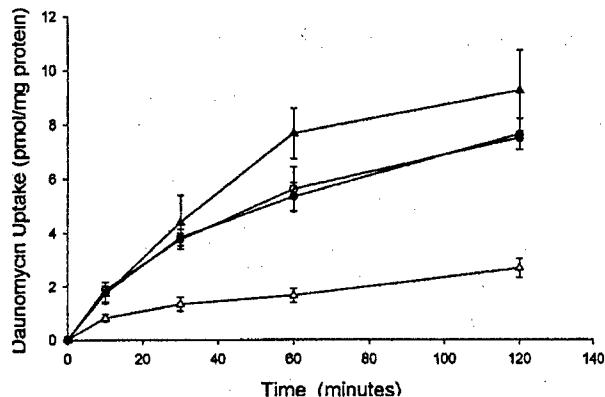


Fig. 2. Time course of daunomycin uptake in MCF-7 sensitive and resistant cells. DNM (0.05 μM) uptake was measured in the presence and absence of verapamil. (▲) MCF-7/ADR + verapamil (100 μM), (Δ) MCF-7/ADR control, (●) MCF-7/WT + verapamil (100 μM), (○) MCF-7/WT control. Data are mean \pm SD of data from one representative study. The study was repeated with similar results.

VBL Accumulation

The uptake of VBL was examined in the presence and absence of ITCs. In MCF-7/ADR cells, verapamil signifi-

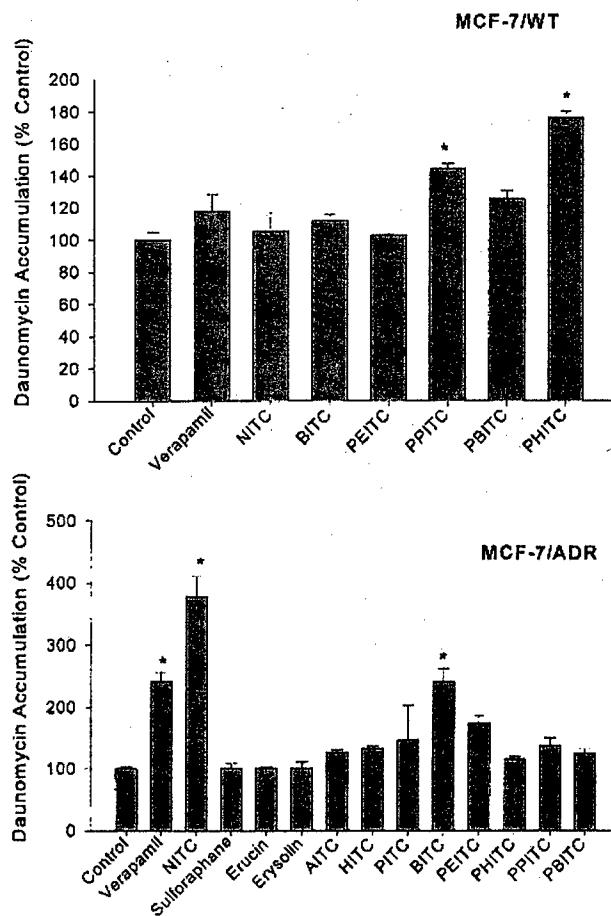


Fig. 3. Effect of organic isothiocyanates (ITCs) on daunomycin accumulation in MCF-7 cells. The 2-h accumulation of 0.05 μM daunomycin was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean \pm SE, $n = 9-12$, * $p < 0.001$.

cantly increased the accumulation of VBL by 33-fold, phenylhexyl ITC by 10-fold, and NITC by 40-fold (Fig. 4). The greatest effects of the ITCs on accumulation were seen for VBL in MCF-7/ADR cells.

PANC-1 Cells

DNM Accumulation

In PANC-1 cells, phenethylisothiocyanate (PEITC), erysolin, NITC, and verapamil were able to significantly increase DNM accumulation (Fig. 5). A number of other ITCs, including BITC, allyl ITC, and hexyl ITC, demonstrated a trend towards increased accumulation of DNM ($p < 0.1$). Concentration-dependent studies demonstrated significant activity for NITC and PEITC at 50 μM concentrations but not at 10 μM concentrations (results not shown).

VBL Accumulation

Verapamil was able to significantly increase VBL accumulation by 4-fold. The ITCs that demonstrated significant effects were: NITC (5.5-fold), PEITC (2-fold), phenylhexyl ITC (3-fold), and phenylbutyl ITC (2.5-fold). All other compounds did not have significant effects, although a number showed a trend towards significance, including BITC, allyl ITC, and hexyl ITC (Fig. 6). The correlation between ITC inhibition (percent control values) for DNM and VBL in PANC-1 cells had an r^2 value of 0.37 ($p < 0.05$; not shown.)

We also examined the correlation between ITC inhibition in MCF-7 cells and PANC-1 cells. The ITC-mediated changes in cellular accumulation for both DNM and VBL in MCF-7/ADR and PANC-1 cells were highly correlated with r^2 values of 0.77 for DNM ($p < 0.05$; Fig. 7A) and 0.86 for VBL ($p < 0.005$; Fig. 7B).

DISCUSSION

Drug resistance represents a major cause for therapeutic failure and death in cancer treatment. An important mechanism of this resistance is the enhanced cellular efflux of a wide

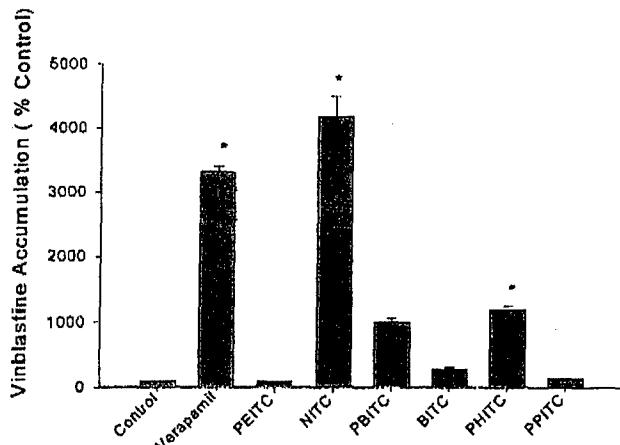


Fig. 4. Effect of organic isothiocyanates (ITCs) on vinblastine accumulation in MCF-7/ADR cells. The 2-h accumulation of 0.05 μM vinblastine was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean \pm SE, $n = 9-12$, * $p < 0.001$.

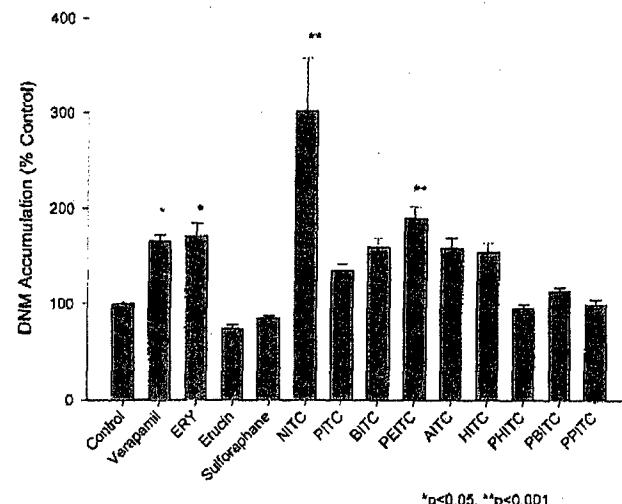


Fig. 5. Effect of organic isothiocyanates (ITCs) on daunomycin accumulation in PANC-1 cells. The 2-h accumulation of 0.05 μM daunomycin was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean \pm SE, $n = 9-12$, * $p < 0.05$ ** $p < 0.001$.

variety of structurally distinct classes of chemotherapeutic agents because of the overexpression of P-gp and/or MRP1. Studies of biopsy samples from patients have revealed elevated levels of P-gp in tumors of every histologic type, with a strong association in leukemias, lymphomas, and some childhood solid tumors between the detection of tumor P-gp and poor response to therapy (15). MRP1 has been identified in a number of different cancers (16): in neuroblastoma, MRP1 levels are elevated and are significantly correlated with N-myc, a negative prognostic factor for response to chemotherapy in neuroblastoma patients. Buser *et al.* (17) reported a high prevalence of P-gp in breast cancer tumor tissue: 83% in early breast cancer and 100% in primarily metastatic breast cancer. One strategy for reversing MDR in cancer has been

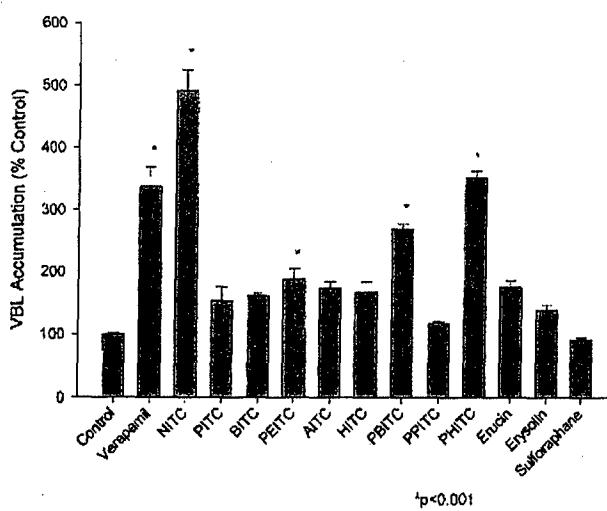


Fig. 6. Effect of organic isothiocyanates (ITCs) on vinblastine accumulation in PANC-1 cells. The 2-h accumulation of 0.05 μM vinblastine was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean \pm SE, $n = 9-12$, * $p < 0.001$.

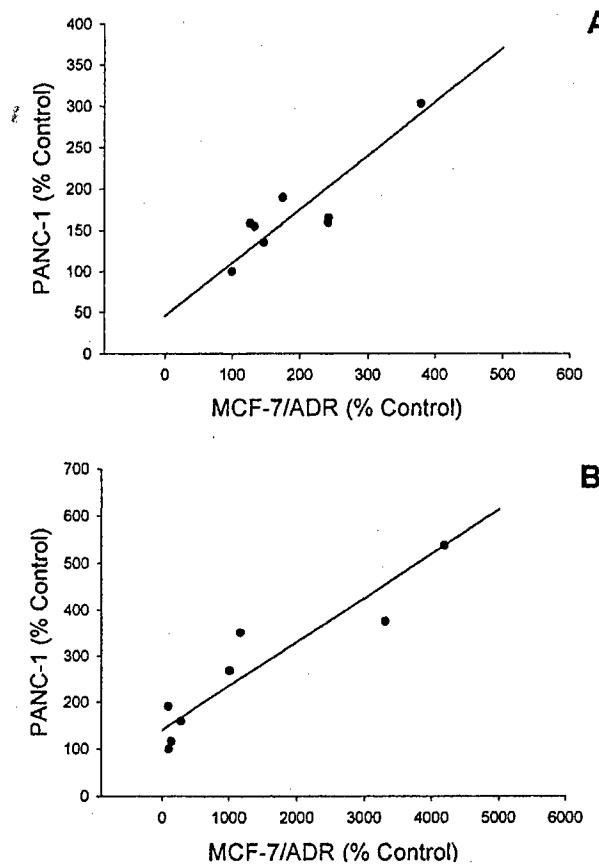


Fig. 7. Correlation between ITC inhibition in MCF-7/ADR cells and PANC-1 cells. (A) The relationship between ITC inhibition of daunomycin in MCF-7/ADR cells with that in PANC-1 cells. $r^2 = 0.77$, $p < 0.05$. (B) The relationship between ITC inhibition of vinblastine in MCF-7/ADR cells with that in PANC-1 cells. $r^2 = 0.86$, $p < 0.005$.

the concomitant use of chemical agents that are by themselves nontoxic but that increase the accumulation of chemotherapeutic drugs in MDR cells through the inhibition of P-gp- or MRP1-mediated efflux of these agents.

In this study, we investigated a class of dietary compounds, the organic ITCs, as inhibitors of P-gp- and MRP1-mediated drug resistance in cancer cell lines. The organic ITCs are components present in the diet, especially in cruciferous vegetables such as broccoli, watercress, cabbage, and brussel sprouts. Numerous experiments have reported that ITCs can inhibit tumor formation of the skin, lung, colon, and breast in animal models (9,18,19), although the mechanism by which this happens is still not completely understood. ITCs are currently being evaluated in clinical trials for the prevention of lung cancer (19). There is substantial evidence that the inhibition of tumorigenesis is partly the result of the direct inhibition and/or downregulation of the CYP-450s responsible for carcinogen activation (16). In addition, ITCs can induce phase II enzymes responsible for the detoxification of electrophilic intermediates formed during phase I metabolism (19). Other mechanisms are likely involved in the chemopreventive effects of ITCs: recent studies have indicated that sulforaphane induces cell cycle arrest and apoptosis in HT29 human colon cancer cells (20) and PEITC also induces apoptosis in cells (21).

Although the organic isothiocyanates represent a group of lipophilic natural products, they have not previously been investigated as substrates or inhibitors of P-gp or MRP1. We have found that NITC and BITC can increase the accumulation of DNM and VBL in the drug-resistant human breast cancer cell line MCF-7 without affecting accumulation in sensitive MCF-7 cells. Interestingly, two of the ITCs tested, phenylpropyl ITC and phenylhexyl ITC, significantly increased the accumulation of DNM in the MCF-7/WT cells but not in the MCF-7/ADR cells. The mechanism underlying this interaction is unknown. Additionally, a number of organic ITCs, including NITC and PEITC, increased the 2-h accumulation of DNM and VBL in PANC-1 cells, which overexpress MRP1 but not P-gp. At this time, it is not known whether these compounds represent substrates for P-gp or MRP1 or whether they are only inhibitors. Because the effects occur rapidly, this suggests that the inhibition might involve a direct interaction at the binding site or at an allosteric site that affects the binding of DNM or VBL. P-gp has been reported to have more than one substrate-binding site. Shapiro and Ling (22) reported that P-gp contains three distinct sites for drug binding, one which transports rhodamine 123, a second that transports Hoechst 33342, and a third that is specific for prazosin or progesterone (23). The anthracyclines inhibit rhodamine 123 transport and stimulate Hoechst 33342 transport whereas VBL, actinomycin D, and etoposide inhibit transport of both dyes. This suggests that compounds like DNM may represent a substrate for only one site whereas VBL may be a substrate for more than one site.

Substrates for MRP1 are endogenous and exogenous organic anions that are conjugated by glutathione, glucuronide, or sulfate, including leukotriene C4 (cysteinyl leukotrienes), glutathione disulfide (oxidized glutathione), and steroid glucuronides (17β -estradiol, 17β -D-glucuronide; 7). Natural product chemotherapeutic agents that do not form a glutathione conjugate, such as anthracyclines, vinca alkaloids, methotrexate, fluorouracil, and chlorambucil (24) are also substrates for MRP1. These drugs are likely transported by MRP1 in a GSH-dependent manner, which may involve the cotransport of GSH and the chemotherapeutic agent (24). Dietrich *et al.* (25) have demonstrated the MRP2-mediated biliary excretion of NITC, either as a GSH conjugate or in association with GSH, indicating that it is a substrate for MRP2. Our studies have demonstrated that the inhibitory effects of the ITCs on either DNM or VBL accumulation in MCF-7/ADR and PANC-1 cells are highly correlated. This finding was not unexpected because there is overlap in substrate specificity for these transporters, with many of the natural product chemotherapeutic agents being substrates for both transporters.

Our concentration-dependent studies indicate that the ITCs are not potent direct inhibitors of P-gp- or MRP1-mediated efflux. Concentrations of $50 \mu\text{M}$ of NITC, PEITC, and BITC are effective inhibitors; after a 2-h accumulation study, the compounds were ineffective at a concentration of $10 \mu\text{M}$. However, concentration-dependent effects after prolonged exposures have not been examined. After vegetable consumption, concentrations of ITCs in plasma are likely in the nM range (26), although there have been no studies that have determined blood levels of unchanged ITCs. Blood concentrations of ITCs would be expected to vary because of genetic differences in their metabolism by glutathione-S-

transferase M1 and T1 (GSTM1 and GSTT1). Conjugation with glutathione, followed by further conjugation reactions to form the mercapturic conjugate, represents the major route of elimination of PEITC and BITC. GSTM1 and T1 exhibit genetic polymorphisms: 60% of Chinese subjects and 40–50% of people in a variety of ethnic groups are deficient in the GSTM1 gene whereas 10–30% of Europeans are deficient in the GSTT1 polymorphism (27,28). These subjects would be expected to have higher blood concentrations of ITCs than those with the wild-type enzyme. It has been reported that the protective effect of dietary ITC intake for lung cancer risk among current smokers is greatest in individuals null for both GSTM1 and GSTT1 genotypes (27,28). What might be more relevant than plasma ITC concentrations would be intracellular concentrations. Intracellular concentrations of ITCs have been reported to be much higher than extracellular concentrations: for example, cells exposed to 100 μM concentrations of sulforaphane have intracellular concentrations of 6.4 mM, likely as GSH conjugates (29). The relationship between intracellular concentrations and efficacy has not been evaluated for the ITCs.

P-gp and MRP1 also play important roles in the bioavailability, distribution, and elimination of administered drugs (8). In the kidney, P-gp is highly expressed on the brush border of the proximal renal tubule. Speeg *et al.* (30) have demonstrated the inhibition of renal clearance of colchicine by cyclosporin, suggesting that MDR modulators may alter the renal elimination processes of anticancer drugs by blocking P-gp in kidneys. P-gp and some isoforms of MRP are present in the apical membrane of intestinal epithelial cells, where they can limit the absorption of xenobiotics, and in the canalicular membrane of hepatocytes, where they can affect biliary excretion. For example, oral administration of paclitaxel to wild-type and *mdrla* knockout mice resulted in a 6-fold higher plasma level of paclitaxel in the latter, at least partly as a result of increased bioavailability (31). We found that 100 μM concentrations of NITC, BITC, and PEITC could significantly increase the 2-h accumulation of DNM in the porcine renal cell line LLC-PK1, which expresses low levels of P-gp (Tseng E and Morris ME, unpublished results). Whether this is the result of inhibition of P-gp and/or other transporters is currently unknown. It is likely that exposure to ITCs present in the diet may affect the bioavailability, and possibly disposition, of compounds transported by P-gp and/or MRP1.

The results of this investigation demonstrate for the first time that P-gp and MRP1 activity can be modulated by naturally occurring organic ITCs. Further studies are needed to evaluate the time-dependent nature of this inhibition, and its clinical relevance.

ACKNOWLEDGMENTS

Financial support for this study was provided by grants from the New York State Health Research Science Board (EMPIRE grant), the Susan G. Komen Breast Cancer Foundation, and U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376. We thank Patricia Neubauer, Wei Shi, and Elizabeth Raybon for technical assistance. This work was previously presented, in part, at the American Association of Pharmaceutical Scientists Annual Meeting, November 2–6, 2000, and published in *Pharm. Res. (Suppl)*, 2000.

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Gender Differences in the Membrane Transport of Endogenous and Exogenous Compounds

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Abstract—Gender differences have been well described in pharmacokinetics and contribute to the interindividual variation in drug disposition, therapeutic response, and drug toxicity. Sex-related differences in the membrane transport of endogenous substrates and xenobiotics have been reported in various organs of the body including kidney, liver, intestine, and brain. These gender-related differences in transport

systems could also contribute to interindividual variability in pharmacokinetics and pharmacodynamics. This review will focus on current knowledge of gender-associated differences in the transport of endogenous and exogenous compounds in a variety of body organs and will discuss the implications and the clinical significance of these observations.

I. Introduction

Gender differences in pharmacokinetics and pharmacodynamics are well documented in animals and humans. Gender is one variable that contributes to differences in pharmacokinetics including absorption, distribution, metabolism, and excretion (Bonate, 1991; Fletcher et al., 1994; Harris et al., 1995). The increased bioavailability of ethanol after oral administration has been reported in women as a result of higher alcohol absorption due to lower gastric alcohol dehydrogenase

activity (Fletcher et al., 1994; Harris et al., 1995), and aspirin is absorbed more slowly in men than in women after oral dosing (Harris et al., 1995). The effect of gender on hepatic metabolism has been extensively examined for a number of drugs (Bonate, 1991; Fletcher et al., 1994; Harris et al., 1995). The enzyme, cytochrome P-450 3A4 (CYP 3A4) is involved in the metabolism of over 50% of drugs in clinical use including erythromycin, lidocaine, and midazolam and is also responsible for the hydroxylation of steroid hormones. The activity of CYP 3A4 in women is 1.4 times greater than that in men (Harris et al., 1995; Gleiter and Gundert-Remy, 1996). Conjugation reactions also demonstrate gender-related differences. The glucuronidation of diflunisal and paracetamol is higher in men than in women due to higher glucuronosyl transferase activity in men, with no sex-associated differences in sulfation (Gleiter and Gundert-Remy, 1996). Gender-based differences in protein bind-

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Article, publication date, and citation information can be found at <http://pharmrev.aspetjournals.org>.

DOI: 10.1124/pr.55.2.1.

TABLE 1
Gender-associated differences in transport systems in humans

| Compounds | Tissue | Process | Gender Effects | Reference |
|--------------|------------------|---|----------------|--|
| Amantadine | Kidney | Urinary recovery at 48 h Renal clearance/weight ^a | M > F M > F | Wong et al., 1995 Wong et al., 1995 |
| Uric acid | Kidney | Postsecretory reabsorption | M > F | Anton et al., 1986 |
| Rifamycin SV | Liver | Normal & Gilbert's patients | M < F | Gentile et al., 1985 |
| MDR1 (Pgp) | Liver | Protein expression | M > F | Schuetz et al., 1995 |
| Glucose | Skeletal muscles | Uptake | M < F | Nuutila et al., 1995 |
| Chylomicron | Splanchnic | Uptake | M > F | Nguyen et al., 1996 |
| Triglyceride | tissues | | | |
| Palmitate | Splanchnic | Uptake | M > F | Nguyen et al., 1996 |

^a Renal clearance in males was greater than in females following normalization for body weight, body surface area, or body mass index.

ing have been observed for diazepam, chlordiazepoxide, and imipramine, with nonpregnant women having higher unbound fractions of these drugs compared with men (Harris et al., 1995; Kashuba and Nafziger, 1998). This may be due to the slightly lower concentrations of α -1-acid glycoprotein and lipoprotein reported in women; the plasma concentration of α -1-acid glycoprotein is decreased by estrogen (Beierle et al., 1999). Gender-related differences in drug response have not been extensively studied; however, a gender effect in pharmacodynamics has been well described for psychotropic drugs. The greater improvement and more severe adverse effects in response to antipsychotic drugs such as chlorpromazine and fluspirilene have been reported in women, at least in part, due to differences in estrogen concentrations; estrogen has been shown to act as a dopamine antagonist (Fletcher et al., 1994; Harris et al., 1995). As well, Kaasinen et al. (2001) have reported that women have significantly higher dopamine D2-like receptor binding than men in the frontal cortex, which may contribute to gender-related differences in the incidence, clinical course, or treatment response in neuropsychiatric diseases that are associated with dopaminergic neurotransmission. There is a gender difference in the response to the cholinesterase inhibitors, rivastigmine and physostigmine, in that female rats exhibit a greater inhibition of cholinesterase in the cerebral cortex, hippocampus, and striatum compared with male rats: orchidectomy completely abolished the difference suggesting that a testicular hormone may be suppressing the effect of the cholinesterase inhibitor by affecting its brain uptake or its interaction with cholinesterase (Wang et al., 2000). Women on hemodialysis exhibit lower responses to recombinant erythropoietin than male patients (Ifudu et al., 2001). It is not known if these differences are due to inherent biological differences in response to erythropoietin or due to other factors including differences in endogenous erythropoietin levels (Ifudu et al., 2001). More adverse effects for antihypertensive drugs are reported in women than in men (Harris et al., 1995). The gender-related differences in pharmacokinetics and pharmacodynamics may explain, at least in part, the interindividual variations observed in drug disposition, therapeutic response, and drug toxicity and are of particular concern for those drugs with relatively

narrow therapeutic ranges (Harris et al., 1995; Gleiter and Gundert-Remy, 1996).

Facilitated transport systems in the intestine, liver, and kidney have been known to play important roles in the absorption and elimination of a variety of clinically significant drugs (Zhang et al., 1998). Drugs must traverse across biological membranes via simple diffusion or physiological transporters to produce therapeutic efficacy (Levy, 1998). Gender-associated differences in transport processes for endogenous and exogenous substrates have been reported in various organs of the body, including kidney, liver, intestine, and brain, for rats, mice, and humans (Kleinman et al., 1966; Orzes et al., 1985; Anton et al., 1986; Morissette et al., 1990; Uhland-Smith and DeLuca, 1993; Sibug et al., 1996). Table 1 summarizes the gender-associated differences in transport activities in humans, evaluated predominantly in clearance studies, whereas Table 2 summarizes the literature information regarding gender differences in transporter mRNA and/or protein expression in tissues. This review will focus on recent knowledge of gender-associated differences in the transport of endogenous compounds and xenobiotics in a variety of body organs and will discuss the implications and the clinical significance of these findings.

II. Membrane Transport in Tissues

A. Kidney

There are gender differences in renal handling of both organic and inorganic anions and cations.

1. Anions. The renal clearance of *p*-aminohippurate (PAH¹) is decreased in female rats due to decreases in both the filtered and secreted amounts. In females, the maximal uptake (V_{max}) into kidney basolateral membrane vesicles is decreased by $52 \pm 9\%$ ($p < 0.05$), and

¹Abbreviations: PAH, *p*-aminohippurate; Oatp, organic anion transporter polypeptide; TEA, tetraethylammonium; rOCT, rat organic cation transport protein; hOCT, human organic cation transport protein; BBM, brush-border membrane; BLM, basolateral membrane; BSP, sulfobromophthalein; TBS, tetrabromosulfonephthalein; BSP-GSH, glutathione conjugate of sulfobromophthalein; Ntcp, sodium-dependent taurocholate transporter; cLPM, canalicular liver plasma membrane; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; DA, dopamine; BBB, blood-brain barrier; 5-HT, 5-hydroxytryptamine; FATP-1, fatty acid transport protein-1.

TABLE 2
Gender differences in transporter expression

| Transport Protein | Typical Substrates | Gender Difference (Species/Tissue) | Sex-Hormone Treatment | | Reference |
|--------------------|--|---|-----------------------|-------------------|--|
| | | | Testosterone | Estradiol | |
| Oatp mRNA/protein | Bromosulfophthalein, taurocholate, ouabain, cortisol, dexamethasone, ajmalinium | Male > female (rat/kidney) male = female (rat/liver) | Strong increase | Decrease | Lu et al., 1996; Simon et al., 1999 |
| rOAT1 protein | PAH, PGE ₂ , urate, salicylate, methotrexate, cAMP, indomethacin, folate | Male > female (rat/kidney) | N.D. | N.D. | Cerrutti et al., 2002 |
| rOCT1 mRNA | Choline, dopamine, epinephrine, serotonin, norepinephrine, MPP, NMN, tyramine | None (rat/kidney) | No effect | No effect | Urakami et al., 1999, 2000 |
| rOCT2 mRNA/protein | Amantadine, TEA, choline, dopamine | Male > female (rat/kidney) | Increase | Moderate decrease | Urakami et al., 1999, 2000 |
| rOCT3 mRNA | Dopamine, guanidine, MPP, TEA | None (rat/kidney) | N.D. | N.D. | Urakami et al., 1999 |
| mdrla mRNA | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Female > male (rat/liver) | N.D. | N.D. | Piquette-Miller et al., 1998 |
| mdrlb mRNA | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Male > female (rat/liver) female > male (mouse/kidney) | N.D. | N.D. | Schinkel et al., 1994; Piquette-Miller et al., 1998 |
| Mdr2 mRNA | Phospholipids, cholesterol | Female > male (rat/liver) | N.D. | N.D. | Furuya et al., 1994; Salphati and Benet, 1998 |
| MDR total protein | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Female > male (rat/liver) Male > female (human/liver) | N.D. | N.D. | Schuetz et al., 1995; Piquette-Miller et al., 1998 |
| Ntcp mRNA/protein | Bile acids | Male > female (rat/liver) | Decrease | N.D. | Simon et al., 1996, 1999 |
| FATP-1 mRNA | Long chain fatty acids | Female > male (human/skeletal muscle) | N.D. | N.D. | Binnert et al., 2000 |

N.D., not determined; PGE₂, prostaglandin E₂; MPP, *N*-methyl-4-phenylpyridinium; NMN, *N*-methylnicotinamide.

the Michaelis-Menten constant (K_m) for uptake into kidney brush-border membrane vesicles is increased by $163 \pm 8\%$ ($p < 0.05$), compared with male rats (Cerrutti et al., 2001). Similar results have been noted in older studies using kidney slices. The transport rate of PAH by kidney slices isolated from male rats is higher than that in female rats with higher V_{max} values compared with female rats (Kleinman et al., 1966; Bowman and Hook, 1972) (Table 3). The rate of accumulation of PAH in renal cortical slices of adult male rats is decreased by castration or by blockade of testosterone receptor sites whereas ovariectomy does not increase the transport of PAH in mature female rats. Furthermore, treatment with estradiol in male rats does not reduce renal tubular transport of PAH whereas chronic (repeated) treatment with testosterone stimulates PAH transport in males more than in females. These results indicate the important role of sex hormones in the renal tubular transport of PAH and suggest distinct renal effects of testosterone compared with estradiol (Braunlich et al., 1993). Similar effects have been reported for the renal tubular trans-

port of Diodrast, amino acids, and thiosulfate (as reviewed by Kleinman et al., 1966).

The urinary excretion of zenarestat, an aldose reductase inhibitor, shows remarkable gender differences in rats and mice (Tanaka et al., 1991, 1992), whereas there is no significant difference between male and female dogs and humans (Tanaka et al., 1992) (Table 4). The ratios of the renal clearance of zenarestat to clearance of zenarestat by glomerular filtration are less than one in male rats and substantially greater than one in female rats. After pretreatment of rats and mice with probenecid, an inhibitor of the active secretion of many organic anions, a marked reduction in the urinary excretion of zenarestat is observed in females but not in males. These results suggest that zenarestat is, at least in part, actively secreted in the kidneys of female rats and mice and active renal tubular secretion of this compound is lacking, or negligible, in male rats and mice (Tanaka et

TABLE 4
Sex difference in the excretion of zenarestat in animals and humans^a

| Species | Sex | Urine | Feces |
|---------|--------|----------------|----------------|
| Rat | Male | 1.5 ± 0.4 | 96.2 ± 1.4 |
| | Female | 46.8 ± 6.2 | 51.2 ± 6.4 |
| Mouse | Male | 3.4 ± 0.7 | 94.2 ± 0.8 |
| | Female | 31.3 ± 5.4 | 66.8 ± 5.7 |
| Dog | Male | 11.9 ± 3.4 | 84.4 ± 3.2 |
| | Female | 9.9 ± 3.4 | 88.9 ± 2.6 |
| Human | Male | 17.7 ± 7.3 | |
| | Female | 22.2 ± 8.8 | |

^a Values are the mean \pm S.D. of three animals, 13 male and 12 female humans expressed as percentage of dose. Adapted from Tanaka et al., 1992.

TABLE 3
Maximal transport rate in the kidney for *p*-aminohippuric acid in male and female rats^a

| Sex | No. of Animals | Kidney Weight/100 g b.wt. | T_{max} |
|--------|----------------|---------------------------|-------------------|
| Female | 8 | 0.70 ± 0.01 | $0.26 \pm 0.01^*$ |
| Male | 3 | 0.71 ± 0.02 | $0.41 \pm 0.04^*$ |

* $p < 0.0005$; adapted from Kleinman et al., 1966.

^a Values are the mean \pm S.E.

TABLE 5
Sex differences in urinary excretion of equalen sodium in rats^a

| Parameter | Total Radioactivity after Oral Administration | Unchanged Drug after Oral Administration | Total Metabolites after Oral Administration |
|-------------------|---|---|--|
| Male | | | |
| C_{max} | 43.2 $\mu\text{g}/\text{ml}$ | 34.2 $\mu\text{g}/\text{ml}$ | 11.3 $\mu\text{g}/\text{ml}^c$ |
| AUC | 540 $\mu\text{g} \cdot \text{h}/\text{ml}$ | 397 $\mu\text{g eq} \cdot \text{h}/\text{ml}$ | 143 $\mu\text{g} \cdot \text{h}/\text{ml}^d$ |
| Urinary excretion | 57.4% ^a | 2.1% ^b | 52.2% ^e |
| Female | | | |
| C_{max} | 38.7 $\mu\text{g}/\text{ml}$ | 36.7 $\mu\text{g}/\text{ml}$ | 2.6 $\mu\text{g}/\text{ml}^c$ |
| AUC | 353 $\mu\text{g} \cdot \text{h}/\text{ml}$ | 326 $\mu\text{g} \cdot \text{h}/\text{ml}$ | 27 $\mu\text{g} \cdot \text{h}/\text{ml}^d$ |
| Urinary excretion | 70.4% ^a | 39.5% ^b | 29.9% ^e |

AUC, area under the curve.

^a Oral dose of 20 mg/kg [¹⁴C]equalen sodium. All values are means expressed as micrograms of equalen equivalents (adapted from Sato et al., 2000). Values with the same superscript letter are significantly different, $p < 0.01$.

al., 1991, 1992). In addition, the urinary excretion of zenarestat is decreased in female rats with experimentally induced chronic diabetes mellitus due to a decrease in active secretion, whereas there is an increase in the urinary excretion of the drug in male rats with experimentally induced acute or chronic diabetes, most likely due to a reduction in testosterone levels in diabetic states (Tanaka et al., 1993). Similarly, it has also been reported that there are gender differences in the renal excretion of perfluorooctanoic acid (Hanhijarvi et al., 1982), S-pentachlorophenyl-N-acetyl-L-cysteine (Smith and Francis, 1983), carnitine (Carter and Stratman, 1982), nilvadipine metabolite (M3) (Terashita et al., 1995) and 1-aminocyclohexanecarboxylic acid (Anton et al., 1986). These compounds are rapidly excreted by an active renal secretion in female rats whereas this secretory mechanism appears to be absent or relatively inactive in male rats (Carter and Stratman, 1982; Hanhijarvi et al., 1982; Smith and Francis, 1983; Anton et al., 1986; Terashita et al., 1995).

Equalen sodium, an antiulcer drug, demonstrates a marked sex-related difference in the urinary excretion of unchanged drug and metabolites in rats. The renal clearance of unchanged drug in male rats is 21 times lower than that in female rats, and the urinary excretion of equalen represented 2.1 and 39.5% of the dose in male and female rats, respectively (Sato et al., 2000) (Table 5). Equalen is secreted in the renal tubules by a probenecid-inhibitable process, which can be inhibited by testosterone. Gonadectomized male rats have a similar renal clearance of equalen as female rats, and treatment of gonadectomized rats with testosterone decreased the renal clearance of equalen (Sato et al., 2000) (Table 6).

Sodium/sulfate cotransport in kidney cortex brush-border (BBM) vesicles and sulfate/anion exchange in basolateral (BLM) vesicles, isolated from female and male guinea pig kidneys, have been studied (Lee et al., 1999a). No statistically significant differences in K_m and V_{max} for uptake were found, although uptake values for female animals tended to be greater; the lack of significance may reflect the small number of animals studied ($n = 4$). Sodium/sulfate cotransport is increased in renal epithelial cells in the presence of estrogen (Lee et al., 1999b). Postmenopausal women demonstrate a de-

creased renal reabsorption of sulfate compared with premenopausal women, although this was not reversed by estrogen supplementation (Benincosa et al., 1995).

A significant gender-related difference occurs in the renal reabsorption of urate in humans, which is of clinical significance. A significant decrease in tubular urate postsecretory reabsorption in the kidneys of adult women leads to a greater urinary excretion and lower serum urate concentrations compared with adult men. Presecretory reabsorption and tubular secretion of urate are similar in women and men. The mechanism underlying this difference is not known but both the renal handling of uric acid and the serum urate levels are not influenced by plasma 17 β -estradiol concentrations (Anton et al., 1986).

Renal organic anion transporting polypeptide (oatp) mRNA expression is higher in male rat kidney than in female kidney and has been shown to be under the control of androgen and to a lesser extent estrogen (Lu et al., 1996). It is speculated that the regulation of kidney oatp expression may be necessary for modulating the renal tubular secretion of conjugated estradiol. Five forms of oatp are expressed in rat kidney. Oatp1 has a wide substrate specificity and substrates include conjugated and unconjugated bile acids, steroid hormones, organic anions such as bromosulfophthalein, and bulky organic cations such as *N*-(4,4-azo-*n*-pentyl)-21-deoxy-*ja*malinium. OATs are multispecific organic anion trans-

TABLE 6
Gender-related differences in renal clearance of equalen sodium in rats^a

| | Unchanged Drug |
|-----------------------------------|-----------------|
| Male | |
| Renal clearance | 0.009 (0.002) |
| Probenecid tx | 0.081 (0.005) |
| Gonadectomy | 0.158 (0.024) |
| Probenecid tx after gonadectomy | 0.055 (0.004) |
| Testosterone tx after gonadectomy | 0.004 (0.002) |
| Female | |
| Renal clearance | 0.193 (0.030)** |
| Probenecid tx | 0.081 (0.009) |
| Gonadectomy | 0.272 (0.055) |
| Testosterone tx after gonadectomy | 0.102 (0.033) |

** $p < 0.01$. tx, treatment.

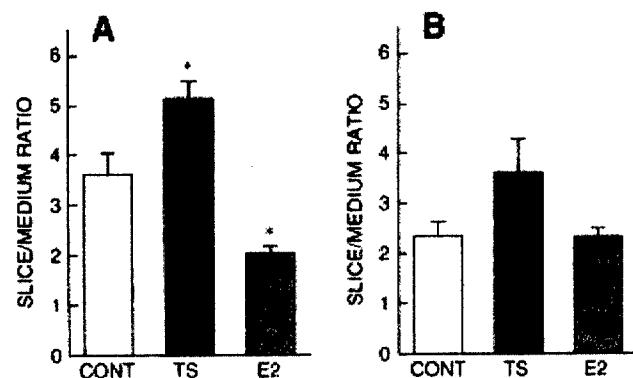
^a IV infusion: rate = 4 $\mu\text{g}/\text{min}$. Results expressed as mean (S.E.) (adapted from Sato et al., 2000).

porters, with all members of the OAT family expressed in the kidney (Sekine et al., 2000). The substrates include endogenous compounds such as prostaglandins, urate and dicarboxylic acids, as well as organic anion drugs including PAH, salicylate, enalapril, and penicillin G (Dresser et al., 2001). Urakami et al. (1999) reported no significant gender-related differences in rat kidney organic anion transporter 1 (rOAT1) mRNA, but Cerrutti et al. (2002) found a significantly lower level of rOAT1 protein expression in rat kidney cortex BLM in females (40% compared with males). The lower expression of rOAT1 in kidney cortex BLM may be responsible, at least in part, for the decreased PAH secretion observed in female rats. Additionally, kidney cortex BBM isolated from female rats exhibit an increased membrane fluidity compared with BBM from male rats (Cerrutti et al., 2002); this may also contribute to the gender differences in membrane transport of substrates.

2. Cations. Tetraethylammonium (TEA) accumulation into renal cortical slices from male rats is significantly greater than that from female rats, suggesting a gender difference in the active secretion of hydrophilic organic cations (Bowman and Hook, 1972). TEA uptake into kidney slices from male and female rats is significantly increased with testosterone treatment; estradiol treatment decreased TEA uptake in kidney slices from male rats but not female rats (Urakami et al., 2000) (Fig. 1). The apparent K_m for distal tubular amantadine transport in female rats is significantly higher than that in male rats whereas the value for amantadine transport in isolated proximal tubules is not different in male and female rats. In addition, apparent V_{max} estimates for amantadine uptake in proximal tubules and distal tubules are not significantly different between males and females (Wong et al., 1993). However, a small number of rats were used in this study and significant differences in transport may have been missed.

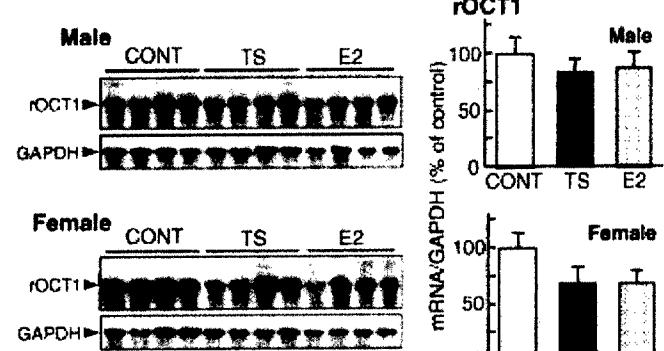
Rat organic cation transport proteins (rOCT) are present in the kidney and are responsible for the transport of a number of organic cations, including TEA, N^1 -methylnicotinamide, choline, and dopamine. Expression levels of rOCT2 mRNA and protein in the male rat kidney are much higher than in females; there was no difference in rOCT1 or rOCT3 expression (Urakami et al., 1999, 2000). Treatment of male and female rats with testosterone significantly increased the expression of rOCT2 mRNA and protein in kidney and increased the TEA accumulation in kidney slices. Estradiol treatment produced a moderate decrease in kidney rOCT2 and decreased TEA accumulation in kidney slices from male, but not female, rats. Testosterone and estradiol treatment had no effect on rOCT1 mRNA or protein expression (Fig. 1). The authors suggest that OCT2 may have a physiological role in the secretion of endogenous substances. Other transporters may also play a role in the kidney transport of TEA.

Part A

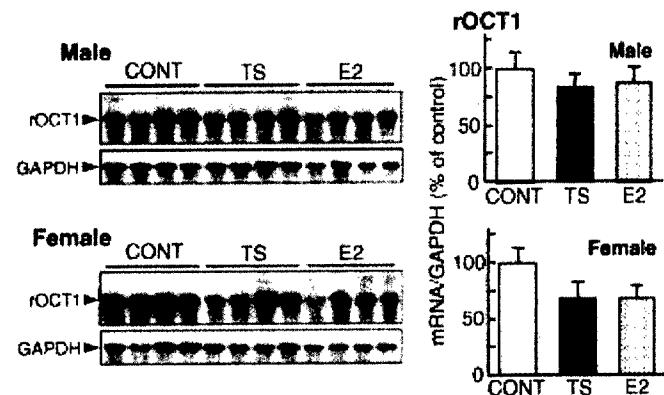


Part B

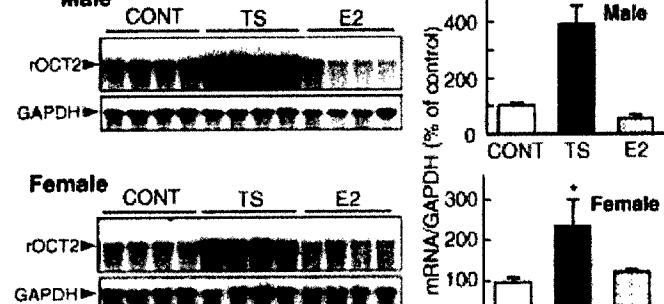
A



B



Male



Female

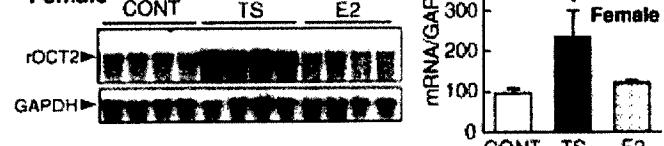


FIG. 1. TEA accumulation by kidney slices from male and female rats treated with testosterone and estradiol. Part A, kidney slices from males (A) and females (B) were incubated at 25°C in buffer containing 50 μ M [14 C]TEA for 60 min. CONT, rats treated with vehicle; TS, rats treated with testosterone; E2, rats treated with 17 β -estradiol. Each column represents the mean \pm S.E. of three separate experiments. *, $p < 0.05$, significantly different from control. Part B, Northern blot analysis of total RNA of the kidney from male and female rats from CONT, TS, and E2 groups. Densitometric quantitation of rOCT1 and rOCT2 mRNA is corrected for loading using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each column represents the mean \pm S.E. of four rats. *, $p < 0.05$. Reprinted with permission (Urakami et al., 2000).

P-glycoprotein (Pgp), present in the brush-border membrane of proximal tubule cells in the kidney, is involved in the renal elimination of a diverse range of lipophilic organic cations. Schinkel et al. (1994) reported a 1-fold higher expression of *mdr1b* in kidney isolated from female mice compared with male mice. The gene products of *mdr1a* and *mdr1b* (in mice) and MDR1 (in humans) are involved in xenobiotic transport and responsible for the multidrug resistance associated with Pgp overexpression in cancer cells. Potential gender differences in the kidney levels of Pgp in humans have not been examined; nor is there information regarding sex hormone effects on Pgp expression in the kidney. However, estrogen and progesterone may be important in the regulation of Pgp function; mRNA and protein expression for Pgp are greatly increased in the secretory luminal and glandular epithelium of the gravid murine uterus, suggesting regulation by the changes in estrogen/progesterone that occur in pregnancy (Arceci et al., 1990).

In clinical studies, the quinidine- and quinine-induced inhibition of renal amantadine clearance occurs only in healthy male subjects (Gaudry et al., 1993) and the urinary recovery at 48 h and the weight normalized renal clearance of amantadine are significantly higher in men than in women (Wong et al., 1995). The human organic cation transporters hOCT1, hOCT2, and hOCT3 have been cloned. hOCT2 is mainly expressed in the kidney but there is no information available regarding gender differences in expression (Dresser et al., 2001).

With regard to inorganic cations, the transepithelial calcium and magnesium reabsorption in the mouse cortical thick ascending limb of Henle's loop is greater in male than female animals, at both 4 and 8 weeks of age; there were no gender-related differences in NaCl transport (Wittner et al., 1997). There are sex differences in the uptake of inorganic mercury into kidney and motor neurons of mice. The uptake of mercury into the female kidney is much lower than that into the male kidney whereas inorganic mercury uptake by female motor neurons is 1.7 times greater than that in males. A smaller accumulation of mercury in the kidney of female mice may result in more circulating mercury which is available to enter muscle and taken up by distal motor axons (Pumphlett et al., 1997).

B. Liver

1. Anions. Gender-associated differences in hepatic transport have been described for organic anions such as sulfobromophthalein (BSP), thymol blue, bilirubin, indocyanine green, tetrabromosulfonephthalein (TBS), and fatty acids. These organic anions are transported to a greater extent into hepatocytes isolated from the livers of female rats than male rats (Orzes et al., 1985; Sorrentino et al., 1988; Torres, 1996).

Marked differences have been reported for the hepatic uptake of a low concentration of BSP between male and

female rats, both in intact animals and in isolated liver preparations and hepatocytes. The uptake rates of BSP in perfused livers, as well as the fractional plasma BSP disappearance rate, are significantly higher in females than in males. The kinetic constants of the low affinity sites are not different between genders whereas the K_m of the high affinity uptake sites in females is significantly lower than that in males with no difference in V_{max} , suggesting that this may be due to a different structural arrangement of the transporter or to a different membrane environment at the sinusoidal domain (Orzes et al., 1985). TBS liver uptake rate in vivo, as well as in sinusoidal liver membrane vesicles, is greater in female rats. V_{max} values for TBS uptake in the membrane vesicles are similar between male and female rats while K_m values in males are significantly higher than that in females (5.5 ± 0.4 versus $17 \pm 4 \mu\text{M}$) (Fig. 2), suggesting that a difference in membrane transport rates may explain the greater accumulation or uptake of TBS in female hepatocytes (Torres, 1996).

Uptake of the glutathione conjugate of sulfobromophthalein (BSP-GSH) at steady state in single-pass liver perfusion studies is increased in female livers compared with male livers. The apparent V_{max} is 48% larger in females whereas the apparent K_m is similar in both sexes. The ratio of influx to efflux, which determines the equilibrium partition of BSP-GSH between the hepatocyte cytosol and plasma compartments, is significantly greater in females with no sex difference in the rate constant of biliary excretion. It has been suggested that these findings indicate that a less negative plasma membrane electrical potential in female livers may provide a more favorable electrochemical driving force for the

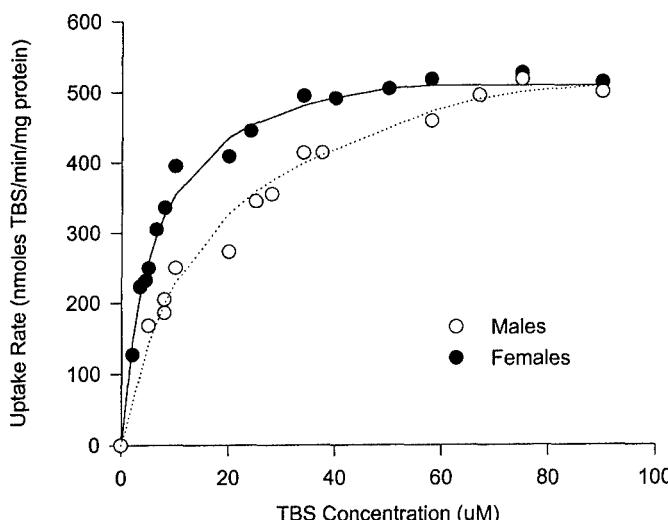


FIG. 2. Kinetics of TBS uptake in sinusoidal liver plasma membrane vesicles from male and female rats. Both curves represent the result of a typical experiment. V_{max} values for TBS uptake are comparable for male and female rats (581 ± 60 versus $544 \pm 15 \text{ nmol/min/mg}$ of protein, mean \pm S.D., $n = 3$); however, the K_m values for TBS uptake in males are significantly higher than in females (17 ± 4.0 versus $5.5 \pm 0.4 \mu\text{M}$, mean \pm S.D., $n = 3$, $p < 0.05$). Adapted from Torres, 1996 with permission from Elsevier Science.

movement of BSP-GSH into the hepatocytes in females (Sorrentino et al., 1988).

Initial oleate uptake velocity in hepatocytes isolated from female rats is also significantly greater than that from male rats. This may be due to a greater affinity of the transport system for oleate in females since no differences are observed in the V_{max} value for hepatic oleate uptake as well as in the surface expression of plasma membrane fatty acid binding proteins between sexes (Sorrentino et al., 1992). Another fatty acid, palmitate, also exhibits a 2-fold higher steady-state uptake rate in livers of female rats compared with male rats (Luxon et al., 1998). Sex differences in the clearance of palmitate by human hepatocytes have been reported (Pond et al., 1996), with hepatocytes isolated from females exhibiting a 2-fold higher clearance.

Although many organic anions are transported to a greater extent by female hepatocytes, sodium-dependent taurocholate uptake is greater in male rats with a significantly higher V_{max} value reported (Simon et al., 1999). Hepatic uptake of taurocholate, the major bile acid, is mainly mediated by the sodium-dependent taurocholate transporter (Ntcp) and to a lesser extent by Oatp. The initial uptake of sodium-dependent taurocholate uptake is shown over a range of concentrations (Fig. 3A). At every concentration, taurocholate uptake was greater in male hepatocytes. Suggested mechanisms that underlie the increased taurocholate transport in male hepatocytes are the greater expression of Ntcp (2-fold greater for both mRNA and protein levels) and the increased sinusoidal membrane fluidity (Lu et al., 1996). Simon et al. (1999) found that Ntcp, but not Oatp, protein content was significantly greater in males and that the expression of Ntcp was transcriptionally regulated. Hepatic Ntcp mRNA levels from female rats were $54 \pm 4\%$ of the value in males (Simon et al., 1999) (Fig. 3B). Female sinusoidal membranes had decreased fluidity (motional order) compared with male membranes, although bile canalicular membranes were not different. Liver sinusoidal membranes isolated from female rats exhibited changes in their phospholipid/fatty acid composition, in that they had a significantly increased phosphatidylethanolamine-to-phosphatidylcholine ratio. This decreased membrane fluidity in female hepatocytes may be involved in lower hepatic taurocholate uptake in females (Simon et al., 1999).

The reduction in the hepatic transport of rifamycin SV is more pronounced in male patients than in female patients with Gilbert's syndrome. This more pronounced defect in hepatobiliary transport in male subjects may explain, at least in part, the greater frequency of Gilbert's syndrome, a pathological condition characterized by unconjugated hyperbilirubinemia, in males (Gentile et al., 1985).

Gender-related differences in the biliary excretion of the organic anion tartrazine, a food dye, has been reported in the rat (Bertagni et al., 1972). Male and female

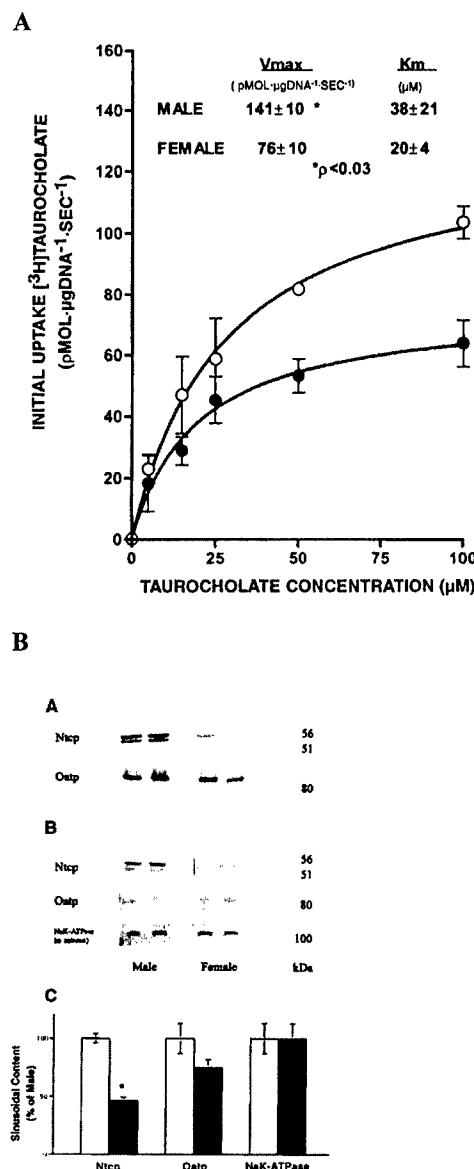


FIG. 3. Saturation kinetics for sodium-dependent taurocholate uptake into isolated rat hepatocytes. A, hepatocytes were isolated from male (○) and oophorectomized (ovx) female (●) rats, and initial uptake of [³H]taurocholate was measured at 60 s in the presence and absence of sodium. These data were used to estimate the maximal uptake (V_{max}) and the Michaelis-Menten constant (K_m) values. Results are means \pm S.E. of three independent experiments in each group. B, immunoblot analysis of sinusoidal membrane proteins from liver homogenates (A) and liver sinusoidal membrane fractions (B), from male and female rats. Liver sinusoidal proteins were identified using specific antibodies. Male (□) and female (■) hepatic steady-state levels of Ntcp, Oatp, and Na⁺-K⁺-ATPase are shown in panel C. Male levels were set at 100%. Results are mean \pm S.E. of 4 to 12 separate determinations. *, $p < 0.01$. Reprinted with permission (Simon et al., 1999).

rats excrete 13 and 29%, respectively, of an intravenous dose of tartrazine by biliary excretion. Treatment of male rats with estradiol increased the excretion from 14 to 33% of the dose, although treatment of female rats with testosterone decreased the biliary excretion from 31 to 16%. Gender-related differences in the biliary excretion of S-ketoprofen have also been reported (Palylyk and Jamali, 1994). In male rats, the major route of elimination is by biliary excretion of the glucuronide

conjugate, whereas in the female rat, the major route of elimination is renal clearance of the conjugate. This results in a marked difference in the amount of *S*-ketoprofen glucuronide eliminated in the urine in female and male rats. There are gender differences in the ATP-dependent canalicular transport of dinitrophenyl-glutathione conjugate (Srivastava et al., 1999). Transport is higher in membrane vesicles isolated from male mice compared with female mice. Additionally, whereas only one transport system is present in male mouse cLPM for the transport of dinitrophenyl-glutathione, there is both high and low affinity systems present in cLPM isolated from female mice. The ATP-dependent transport of organic anions, including glucuronide and glutathione conjugates, occurs by multidrug resistance-associated protein 2 (MRP2), also known as the canalicular multispecific organic anion transporter (cMOAT). MRP2 is the major transporter responsible for secretion of bilirubin glucuronides into bile; gender differences in the expression of MRP2 have not been examined.

2. Cations. Pgp is present on the canalicular membrane of hepatocytes and involved in the biliary excretion of phospholipids, cholesterol, and a wide variety of lipophilic organic cations. Hepatic expression of the gene product of *mdr2* in female rats is 7-fold higher than in male rats (Furuya et al., 1994). This isoform of Pgp is mainly involved in phospholipid transport across the canalicular membrane. Piquette-Miller et al. (1998) and Salphati and Benet (1998) reported higher levels of total *mdr* gene products in female rat livers compared with male livers. Gender differences in *mdr* mRNA levels were also seen. Male livers contained more than 2-fold higher levels of *mdr1b* and female livers contained higher levels (approximately 35–50%) of *mdr1a* and *mdr2* (Piquette-Miller et al., 1998; Salphati and Benet, 1998). In humans, hepatic Pgp (total) protein expression is 2-fold higher in men than in women (Schuetz et al., 1995), suggesting that the drug disposition of Pgp substrates could be different between genders, resulting in differences in drug efficacy and toxicity between males and females. Interestingly, there are gender-related differences in Pgp expression and functional activity in peripheral blood samples of subjects with B-type chronic lymphocytic leukemia, with significantly more men (89%) than women (48%) being MDR1 phenotype-positive (Steiner et al., 1998). These findings are consistent with the overall better prognosis for women with chronic lymphocytic leukemia than for men (Steiner et al., 1998).

C. Intestine

Very little is known regarding gender-related differences in intestinal uptake and drug bioavailability. Clinical studies have reported an increased bioavailability of both iron and ethanol in women but these gender-related differences likely do not involve differences in intestinal transporters. For ethanol, the increased bio-

availability is likely due to decreased gastric metabolism of alcohol in women (Lieber et al., 1994). Decreases in both the rate and extent of absorption of acetaminophen occur in late pregnancy; this is likely due to decreases in the rate of gastric emptying (Galinsky and Levy, 1984).

A gender-related difference has been documented in the transport of calcium in the intestine. Kinetic analysis of calcium transport across the rat intestine has shown that there are two transport processes, one of which is saturable and the other nonsaturable. The saturable transport process is regulated by vitamin D and is predominantly located in the proximal intestine whereas the nonsaturable process is not vitamin D-dependent and has similar capacity throughout the intestine (Bronner et al., 1986). Intestinal calcium transport is significantly greater in male rats than in female rats in a vitamin D-sufficient condition, although it is comparable between sexes in the presence of vitamin D deficiency. Vitamin D deficiency produces a markedly lower intestinal transport of calcium in male rats but not in female rats. This observation suggests that calcium transport in the intestine of female rats, unlike male rats, is mediated by a vitamin D-independent mechanism at the calcium intake levels studied in this investigation (Uhland-Smith and DeLuca, 1993).

Total intestinal absorption of calcium is enhanced during pregnancy and lactation in vitamin D-deficient rats. Intestinal calcium absorption during the rat estrous cycle is highest during estrus and lowest during diestrus following the administration of both high and low calcium diets. Since the highest serum levels of estradiol, progesterone, prolactin, follicle-stimulating hormone, and luteinizing hormone are present during estrus (Butcher et al., 1974; Brommage et al., 1990), the greatest intestinal absorption of calcium observed during estrus may be related, either directly or indirectly, to any one of several sex hormones (Brommage et al., 1993). Intestinal mucosal cells contain estrogen receptors, and calcium uptake in duodenal cells is significantly enhanced by about 60% by 17 β -estradiol at a concentration of 10 nM (Arjmandi et al., 1993). Administration of 17 β -estradiol at a dose of 40 μ g/kg b.wt./day for 21 days significantly elevated intestinal absorption of calcium in female rats whereas serum levels of 1,25-dihydroxyvitamin D were unaltered (Arjmandi et al., 1994). These findings suggest that transluminal calcium uptake is promoted by a direct action of 17 β -estradiol on the intestinal tract with no increase in the circulating levels of 1,25-dihydroxyvitamin D (Arjmandi et al., 1993, 1994).

The implications of these observations are as follows. First, estrogen may play an important physiological role in regulation of intestinal calcium absorption. High estrogen levels during pregnancy and estrus may promote calcium absorption, and estrogen deficiency in menopause may result in calcium malabsorption by a direct action on the intestine. The malabsorption of calcium in

the intestine as a result of ovarian hormone deficiency in postmenopausal women is often associated with osteoporosis characterized by bone loss (Heaney et al., 1978; Gallagher et al., 1979, 1980; Gallagher, 1990). Second, the rate and extent of intestinal calcium absorption may be modulated by compounds that block or mimic estrogen action.

Aluminum, at a concentration of 2 μM , significantly decreases mucosa-to-serosa calcium influx in duodenal everted sacs both of male and female rats compared with aluminum-free controls; however, the percentage of reduction in females (31.2%) is greater than that in males (17.8%). The sensitivity to the inhibitory effect of aluminum on duodenal calcium flux is raised with increasing serum levels of 17β -estradiol in ovariectomized female rats with no alterations in the maximal response, whereas the effect of aluminum on calcium flux in duodenal sacs is not dependent of serum testosterone levels in castrated male rats injected with testosterone. These results demonstrate that there are gender-associated differences in the inhibitory effect of aluminum on trans-luminal calcium transport in the duodenum of the rat (Orihuela et al., 1996).

D. Brain

There have been few studies that have examined the potential for gender-related differences in transport across the blood-brain barrier (BBB). 17β -Estradiol treatment of ovariectomized rats increases 2-deoxyglucose uptake into brain, which is likely due to the increase in the mRNA and protein expression of glucose transporter 1 (GLUT-1) in the BBB epithelium (Shi and Simpkins, 1997). These results support a modulatory role for estrogens in the brain transport of glucose.

Both Pgp and MRP1 are present in the BBB epithelium and are responsible for the active efflux of drugs from the brain, minimizing brain exposure to many organic anions and cations. Although Pgp exhibits gender differences in expression in liver, this has not been examined for the BBB. Gender differences in the BBB uptake of verapamil have been reported in mice where female mice have increased functional Pgp activity, resulting in decreased verapamil influx into the brain (Dagenais et al., 2001). However, gender differences in uptake were not observed for two other Pgp substrates, morphine or quinidine (Dagenais et al., 2001), so the significance of these findings is unknown.

The effect of gender on the reuptake of dopamine (DA) by the sodium-dependent DA transporter into nerve terminals, the primary mechanism for inactivation of DA following its release into the synapse, has been examined. An increased synaptosomal DA reuptake in the anterior hypothalamus is observed in ovariectomized rats treated with estradiol due to an increase in the number of DA uptake binding sites (Cardinali and Gomez, 1977). The maximal binding density (B_{\max}) of striatal DA uptake sites is significantly elevated 15 and 30

min after an injection of a physiological dose of 17β -estradiol in ovariectomized rats with no change in the binding affinity (K_d) of the DA uptake sites. There is no effect of progesterone on striatal DA uptake after progesterone treatment of ovariectomized rats. The increase of DA uptake binding sites by the administration of 17β -estradiol is rapid and short-lasting and is associated with peak 17β -estradiol plasma levels, suggesting most likely a membrane-linked nongenomic effect of 17β -estradiol (Morissette et al., 1990). When ovariectomized rats are chronically treated with 17β -estradiol and/or progesterone at pharmacological doses, DA uptake site density in the striatum is significantly increased by 16 to 23% without an alteration in the binding affinity, most likely due to an increased synthesis of the DA transporter by a genomic effect of these female sex hormones. In addition, chronic exposure to 17β -estradiol and/or progesterone up-regulates the DA uptake sites in the nigrostriatal dopaminergic pathway whereas the nucleus accumbens and the substantia nigra pars reticula are not affected (Morissette and Di Paolo, 1993a). Striatal DA uptake site density is significantly lower in normal male rats, gonadectomized male rats, and ovariectomized female rats compared with normal female rats and fluctuates during the female estrous cycle with a peak occurring in the morning of proestrus when estradiol is elevated and progesterone is low, suggesting an up-regulation of striatal DA uptake sites by estradiol (Morissette and Di Paolo, 1993b). This agrees with the findings of an investigation examining the effect of acute treatment with 17β -estradiol (Morissette et al., 1990). It has also been shown that 17β -estradiol increases DA uptake in mesencephalic neurons isolated from females but not in male neurons, and male sex hormones, testosterone and dihydrotestosterone, have no effect (Engele et al., 1989). In humans, DA and serotonin (5-hydroxytryptamine; 5-HT) transporter availability is greater in females compared with males, as determined by single photon emission computed tomography imaging using an analog of cocaine (CIT) that labels DA and 5-HT transporters (Staley et al., 2001). Therefore, gonadal hormones may play an important role in the effects of psychoactive drugs acting on neuronal DA uptake sites and modulation of the DA transporter by these hormones will represent a source of interindividual variability in the treatment of neuropsychiatric disorders and neurologic diseases such as Parkinson's disease (Cardinali and Gomez, 1977; Engele et al., 1989; Morissette et al., 1990; Morissette and Di Paolo, 1993a,b).

A sexual dimorphism in the density of norepinephrine transporters has been demonstrated in the frontal cortex of rats, with males having significantly fewer binding sites than females, whereas the binding affinity of the uptake sites was not different between genders (Vathy et al., 1997). 5-HT uptake in the anterior and middle hypothalamus of intact female rats exceeds sig-

nificantly that in intact male rats (by about 30–40%) and is similar to that in neonatally castrated adult rats (Fig. 4), suggesting that androgens may play a key role in the development of the hypothalamic serotonergic system over the neonatal period by inhibiting either the serotonergic axon ingrowth to the hypothalamus or the ramifications of the axonal terminal portions (Borisova et al., 1996). Estradiol treatment stimulates a significant increase in the density of 5-HT_{2A} binding sites in the anterior frontal, anterior cingulate and piriform cortex, the olfactory tubercle, the nucleus accumbens and the lateral dorsal raphe nucleus, areas of brain concerned with cognition, emotion, and motor control, suggesting that the antidepressant action of estrogen may be mediated by a serotonergic mechanism (Fink et al., 1996).

E. Other Tissues

The rate of glucose uptake in skeletal muscle, under hyperinsulinemic and normoglycemic conditions, is significantly greater in women than in men (Fig. 5), suggesting an increased sensitivity to insulin in women (Nuutila et al., 1995). Basal and maximal insulin-stimulated glucose transport is also significantly higher in adipocytes isolated from female rats and human female subjects compared with males (Foley et al., 1984). In skeletal muscle, fatty acid transport protein-1 (FATP-1) mRNA levels are higher in lean women than in lean men (2.2 ± 0.1 versus 0.6 ± 0.2 attomoles/ μg of total RNA, $p < 0.01$). FATP-1 mRNA was significantly decreased in skeletal muscle of obese women, but no change in FATP-1 expression was seen in men. Additionally, insulin infusion reduced FATP-1 mRNA in muscle of lean women, but not in men (Binnert et al., 2000). This study indicates that lean women may be able to utilize lipids to a greater extent than men, although whether differences

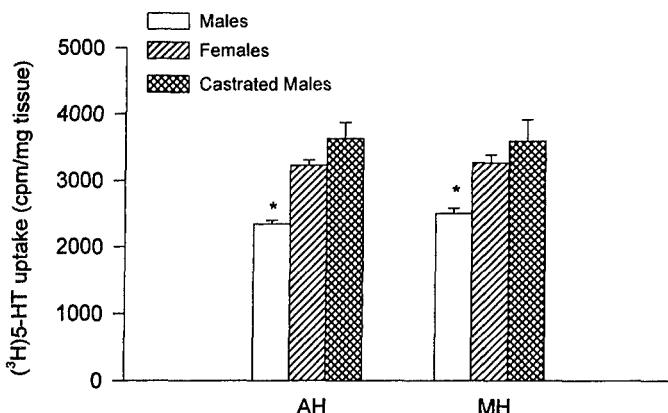


FIG. 4. Specific uptake of radioactively labeled serotonin ($[^3\text{H}]5\text{-HT}$) by the anterior (AH) and middle hypothalamus (MH) in adult male, adult female, and neonatally castrated males. Specific uptake is the difference of $[^3\text{H}]5\text{-HT}$ uptake in the absence and presence of citalopram 10^{-5} M. The columns represent the values for 10 to 25 rats. *, $p < 0.001$ compared with the levels in females and castrated males. Adapted with permission from Borisova et al., 1996.

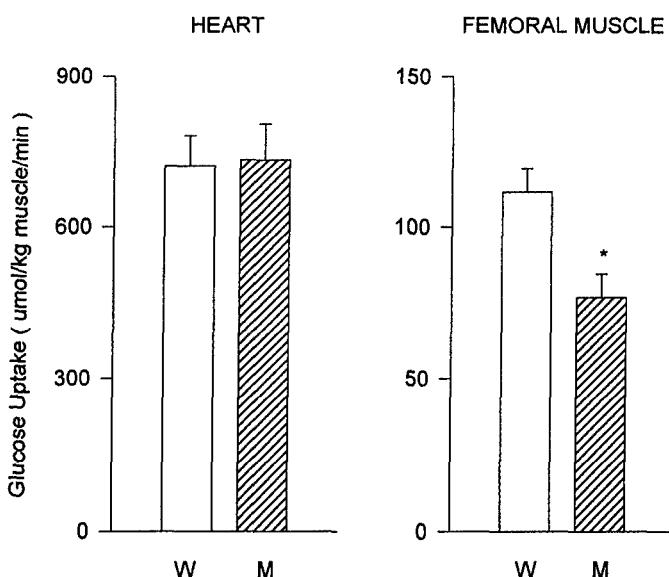


FIG. 5. Rates of glucose uptake in the heart and femoral muscles (micromoles per kilogram of muscle per minute) in normal women (W) and men (M). Insulin sensitivity of glucose uptake was determined in heart and muscle tissues using positron emission tomography under hyperinsulinemic and normoglycemic conditions. *, $p < 0.01$ compared with women. Adapted with permission from Nuutila et al., 1995.

in FATP-1 mRNA result in corresponding differences in FATP-1 protein expression is not known.

A marked difference in the splanchnic uptake of chylomicron triglyceride is observed between men and women. Chylomicron uptake in the splanchnic tissues in men and women accounts for 71% and 20% of meal triglyceride disposal, respectively, indicating greater meal fatty acid storage in visceral adipose tissue in men and gender-specific differences in body fat distribution (Nguyen et al., 1996).

III. Conclusions

Gender differences in the transport of numerous drugs and endogenous substrates exist in animals and humans. Sex-associated differences are described for renal tubular secretion of organic anions and cations, hepatic uptake of taurocholate and organic anions including endogenous compounds, intestinal calcium transport, and Pgp-mediated and neurotransmitter transport in the brain. Gender-related differences in transporter mRNA and protein expression represent an important mechanism for the regulation of hepatic transport processes. Furthermore, female sex hormones, mainly estradiol, and male sex hormones, primarily testosterone, appear to be involved in these gender-related differences in transport either directly or indirectly. In addition, gonadal hormones can be used to treat neurologic diseases and neuropsychiatric disorders by modulating the DA uptake sites in the brain.

Gender differences in membrane transport in humans are not always consistent with differences reported in animal studies. For example, Pgp, an ATP-dependent efflux pump present in cancer cells and excretory or-

gans, demonstrates a higher hepatic expression in men than in women (Schuetz et al., 1995), but opposite changes have been reported in rats (Furuya et al., 1994; Piquette-Miller et al., 1998). In addition, gender differences in the urinary excretion of zenarestat are observed in mice and rats but not in dogs and humans (Tanaka et al., 1992). This emphasizes the importance of performing studies in humans to evaluate the effect of gender.

Gender-associated differences in the nature and prevalence of many diseases may be explained, at least in part, by the differences in the transport processes of substrates between male and female subjects. In addition, these gender-related differences in transport systems may be responsible, at least in part, for interindividual variability in drug disposition, therapeutic response, and drug toxicity. Research is needed to evaluate potential gender differences in regulation, expression, and activity of known transport proteins involved in the uptake or secretion of both endogenous and exogenous compounds.

Acknowledgments. We acknowledge research support for the studies described in this review from National Science Foundation Grants IBN9629470 and IBN9973499 and grants from the Western New York Kidney Foundation/Upstate New York Transplant Services. Support through the Susan G. Komen Breast Cancer Foundation and U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376 is also acknowledged.

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Appendix 3

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CHROMATOGRAPHY B

Journal of Chromatography B, 788 (2003) 17–28

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Determination of α -naphthylisothiocyanate and metabolites α -naphthylamine and α -naphthylisocyanate in rat plasma and urine by high-performance liquid chromatography

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Received 27 August 2002; received in revised form 4 December 2002; accepted 4 December 2002

Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination of α -naphthylisothiocyanate (1-NITC) and two metabolites α -naphthylamine (1-NA) and α -naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Partisphere C₁₈ 5-μm column, a mobile phase of acetonitrile–water (ACN–H₂O 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. The lower limits of quantitation (LLQ) in rat plasma, urine, and ACN were 10, 30, and 10 ng/ml for 1-NITC; 30, 100, and 30 ng/ml for 1-NA; and 30 ng/ml in ACN for 1-NIC. At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the range of within-day and between-day accuracies were 95–106 and 97–103% for 1-NITC in plasma, respectively. Stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at –20 and –80 °C in plasma, urine, and ACN precipitated plasma and urine, but degraded at room temperature and 4 °C. 1-NA was stable in all of the tested matrices at all temperatures. 1-NIC was unstable in plasma, urine, and ACN precipitated plasma and urine, but stable in ACN. The degradation product of 1-NITC and 1-NIC in universal buffer was confirmed to be 1-NA. 1-NITC and 1-NA were detected and quantified in rat plasma and urine, following the administration of a 25 mg/kg i.v. dose of 1-NITC to a female Sprague–Dawley rat.

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Keywords: α -Naphthylisothiocyanate; α -Naphthylamine; α -Naphthylisocyanate

1. Introduction

Many synthetic and naturally occurring organic isothiocyanates (ITCs; RN=C=S) can block chemical carcinogenesis in experimental animals and are being considered as chemopreventive agents for human use

(see reviews in Refs. [1,2]). α -Naphthylisothiocyanate (1-NITC) (Fig. 1) was reported as a carcinogenesis inhibitor in rats as early as the 1960s [3–7]. Recently, we have found that 1-NITC can reverse the multidrug resistance (MDR) to antineoplastic agents in human cancer cell lines through inhibition of the ATP-dependent efflux proteins, P-glycoprotein and multidrug resistance associated-protein 1 (MRP1) [8]. These findings indicated the potential use of 1-NITC not only in cancer preven-

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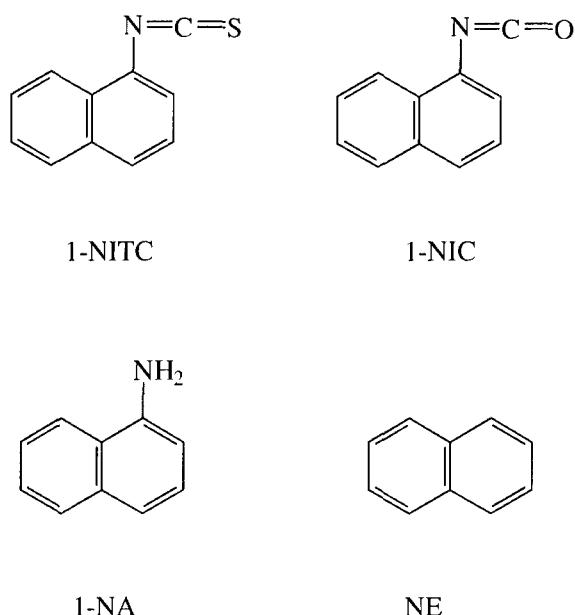


Fig. 1. Chemical structures of 1-NITC, 1-NA, 1-NIC and NE.

tion but as a chemosensitizing agent. High doses of 1-NITC (150–300 mg/kg) produce hepatic lesions resembling those occurring with biliary cirrhosis in humans [9–11]; this has led to the use of 1-NITC as a model chemical to study human cirrhosis and drug-induced cholestasis.

To our knowledge, the pharmacokinetics of 1-NITC and its metabolites in rat plasma and urine have not been studied, although it is reported that 1-NITC is eliminated in the bile and urine predominantly as metabolites [12]. The major metabolites of 1-NITC have been characterized by mass spectroscopy and found to be α -naphthylisocyanate (1-NIC) and α -naphthylamine (1-NA) [16]. Several analytical methods have been described for the determination of 1-NITC in biological samples, including assays based on radiolabelled drug [10–13], thin-layer chromatography [14], gas–liquid chromatography (GLC) [15], gas chromatography/mass spectrometry (GC–MS) [16], and high-performance liquid chromatography (HPLC) [17–22]. However, those methods were labor intensive, time consuming, inconvenient, or not sensitive enough. In this paper we report the development and validation of a rapid and sensitive HPLC assay able to detect the parent drug 1-NITC and its metabolites in rat

plasma and urine, the stability of 1-NITC and its metabolites in rat plasma and urine samples, and the use of the assay to characterize the pharmacokinetics of 1-NITC in a preliminary rat study.

2. Experimental

2.1. Chemicals and reagents

1-NITC and 1-NA were purchased from Sigma (St. Louis, MO, USA) more than 99 and 98% purity, respectively. 1-NIC was purchased from Aldrich (Milwaukee, MI, USA) at 98% purity. The internal standard naphthalene (NE) (Fig. 1) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) at more than 99% purity. Acetonitrile (ACN) and methanol (MeOH) were HPLC grade from Fisher. Other chemicals are in analytical grade unless specified.

2.2. Preparation of rat plasma and urine samples for calibration of standards and quality control samples (QCs)

The stock solutions 10 mg/ml of 1-NITC, 1-NA, 1-NIC, and NE were freshly prepared for every validation run by dissolving a weighted amount of each compound in ACN. The 0.5 and 2.0 mg/ml working solutions of NE were prepared by diluting the stock solution with ACN as internal standard for validation of 1-NITC and 1-NA in rat plasma and urine samples, respectively.

Solutions of 1-NITC containing 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250 μ g/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat plasma sample (50 μ l) was spiked with 5 μ l of a NE solution (0.5 mg/ml), 5 μ l of varying concentrations of 1-NITC, and 190 μ l ACN, to prepare a series of standards (10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml as final concentration) for the calibration curve.

The working solutions of 1-NA containing 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 μ g/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat urine sample (50 μ l) was spiked with 5 μ l NE work solution (2.0 mg/ml), 5 μ l appropriate 1-NA working solution, and added 190 μ l ACN, to prepare a series of standards (100,

200, 500, 1000, 2000, 5000, 10 000, 20 000, and 50 000 ng/ml as final concentration) for the calibration curve.

Both spiked plasma and urine samples were vortexed for 10 s and centrifuged at 10 000 g for 5 min at 4 °C. The resulting supernatants were used for injection. QC samples at low (10 ng/ml for 1-NITC and 100 ng/ml for 1-NA), medium (500 ng/ml for 1-NITC and 5000 ng/ml for 1-NA), and high concentrations (5000 ng/ml for 1-NITC and 50 000 ng/ml for 1-NA), respectively, were prepared by the same procedures as previously described.

2.3. HPLC instrumentation and conditions

The Waters HPLC system (Milford, MA, USA) consisted of a model 1525 binary pump, a model 717plus autosampler (a 250- μ l injector and a 200- μ l loop) configured with a heater/cooler, a model 5HC column oven, and a model 2487 UV detector. The column and autosampler temperatures were kept at room temperature (21 ± 1 °C) and 4 °C, respectively. The reversed-phase chromatography was performed with a Partisphere C₁₈ 5- μ m column 125×4.6 mm I.D. (Whatman, Clifton, NJ, USA) protected by a RP guard cartridge system C₁₈ 5- μ m (Whatman), and eluted isocratically with a mobile phase consisting of ACN–H₂O (70:30, v/v). The flow-rate was 1.0 ml/ml and the injection volume was 50 μ l. The UV detector was set at a single wavelength of 305 nm. The Breeze System software version 3.2 (Waters) was used for instrument control and data analysis.

2.4. Assay validation

2.4.1. Lower limit of quantitation

The lower limit of quantification (LLQ) was determined during the evaluation of the linear range of calibration curve. LLQ was defined as the concentration of the lowest QC samples producing an assayed concentration within 10% of the theoretical value (i.e. accuracy between 90 and 110%) and yielding a precision of more than 90% for both within- and between-day evaluation.

2.4.2. Linearity of calibration curve

The linearity of calibration curve was evaluated by regression analysis of peak area ratios (1-NITC/NE

and 1-NA/NE) to 1-NITC and 1-NA concentrations in blank plasma and urine samples, respectively.

2.4.3. Precision and accuracy

The assay was validated by within- and between-day accuracy and precision quantifying 1-NITC and 1-NA at QCs. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentrations. Within-day variability was assessed through the analysis of QCs in triplicate, and between-day variability was determined through the analysis of QCs on four consecutive days.

2.4.4. Recovery

The recovery of 1-NITC and 1-NA was established with QCs by comparing peak area ratios (1-NITC/NE and 1-NA/NE) to those of standards in ACN. The mean recoveries at low, medium, and high concentrations were determined for both within- and between-day analyses.

2.5. Stability

The stability of 1-NITC, 1-NA, and 1-NIC was studied in different matrices consisting of rat plasma, urine, ACN precipitated plasma and urine, ACN, and in a universal buffer (citrate–phosphate–borate–HCl, pH 2–12) at four designated temperatures over 96 h. 1-NITC, 1-NA, or 1-NIC (200 ng/ml as final concentration), along with internal standard NE (10 μ g/ml), were added to plasma and ACN, respectively, for stability evaluations in plasma, ACN precipitated plasma, and ACN samples at room temperature (RT), 4, –20, and –80 °C. Samples were assayed at time points up to 96 h. The stability of 1-NITC, 1-NA, and 1-NIC in urine and ACN precipitated urine were tested at similar time intervals up to 96 h at a final concentration of 10 μ g/ml for 1-NITC, 1-NA, and 1-NIC and 50 μ g/ml for NE. The stabilities of 1-NITC, 1-NA, and 1-NIC in universal buffer were determined over a pH range from 2 to 12 at RT at times up to 96 h using the same concentrations as used for plasma samples. The compound was considered stable if the variation of

quantitation was less than 10% (i.e. 90–110% of initial time concentration).

2.6. 1-NITC pharmacokinetics in rat

The jugular vein cannula was inserted into a female Sprague–Dawley (Harlan, Indianapolis, IN, USA) rat following an i.m. injection of ketamine 90 mg/kg and xylazine 10 mg/kg (Henry Schein, Melville, NY, USA). Three days following surgery, a dose of 25 mg/kg 1-NITC (10 mg/ml) in a vehicle consisting of 10% ethanol (Pharmaco Products, Brookfield, CT, USA), 10% cremophor EL (Sigma), and 80% sterile saline (Braun Medical, Irvine, CA, USA) solution was administered as an intravenous (i.v.) bolus through the cannula.

Blood samples (250 µl each) were collected at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, and 24 h following 1-NITC administration, and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma was immediately separated from blood via centrifugation at 1000 g for 10 min at 4 °C and stored at –80 °C to prevent potential degradation of 1-NITC and metabolites. The internal standard (5 µl) was added to 50 µl of each plasma sample and treated as previously described. The data was fitted to obtain pharmacokinetic (PK) parameters using WinNonLin version 2.1 (Pharsight, Mountain View, CA, USA).

Urine samples were collected at 2, 4, 6, 9, 12, 24, and 25 h time points, and the volume was measured. After adding 0.1% sodium azide (Fisher), the urine samples were centrifuged at 1000 g for 10 min at 4 °C and stored at –80 °C to prevent potential degradation of 1-NITC and 1-NA. Five µl NE (2.0 mg/ml) was added to 50 µl of each urine sample before assay.

3. Results

3.1. Specificity and selectivity

Figs. 2 and 3 display typical chromatograms resulting from HPLC analysis of the ACN precipitated rat plasma and urine. Blank rat plasma and urine do not demonstrate any interference peaks (Figs. 2a and 3a). The mixture of 1-NITC, 1-NA and 1-NIC (200 ng/ml each) and internal standard in

ACN solution are well separated from one another with retention times (t_R) of 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.6 min) (Fig. 2b). The rat plasma and urine samples spiked with 1-NITC, 1-NA, 1-NIC and NE standards show similar results (Figs. 2c and 3b), except that 1-NIC is absent due to possible rapid degradation in plasma and urine samples (Figs. 2d and 3c). 1-NITC, 1-NA, and NE are separated well from potentially interfering endogenous plasma and urine compounds under the current optimal chromatographic conditions (Figs. 2a,c,d, and 3a–c). In biological samples obtained after the i.v. administration of 1-NITC to a rat, 1-NITC and 1-NA were the only compounds that could be detected in plasma (Fig. 2e) and urine (Fig. 3d), respectively.

3.2. Lower limit of quantitation (LLQ)

The LLQ of 1-NITC, 1-NA, and 1-NIC was determined in blank rat plasma and urine samples, as well as in ACN solution. As shown in Table 1, the lower limit of quantitation (LLQ) of 1-NITC, 1-NA, and 1-NIC are dependent on the matrix. The LLQ of 1-NITC is 10 ng/ml for plasma and ACN samples, and 30 ng/ml for urine samples. The LLQ of 1-NA is about three-fold more than 1-NITC, i.e. 30 ng/ml for blank rat plasma and ACN, and 100 ng/ml for blank rat urine. 1-NIC can be detected only in ACN with a LLQ of 30 ng/ml.

3.3. Linearity

The linear regression correlation coefficient r was more than 0.999 in every standard curve (data not shown). The linearity for 1-NITC and 1-NA was tested over a concentration range of 10–5000 ng/ml and 30–5000 ng/ml, respectively, in rat plasma. For rat urine samples, the calibration curves of 1-NITC and 1-NA were linear over the concentration range of 30–5000 and 100–50 000 ng/ml, respectively.

3.4. Accuracy, precision and recovery

As shown in Table 2, at low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of 1-NITC, the within- and between-day accuracy were 95–106 and 97–103%, respectively. The with-

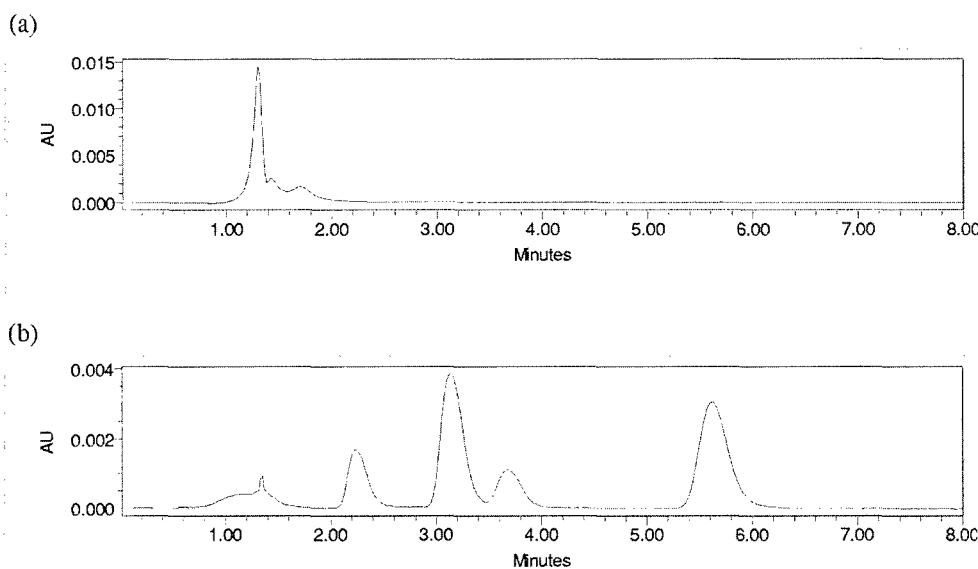


Fig. 2. Typical chromatograms for rat plasma samples obtained from the analysis of (a) blank plasma. (b) ACN containing 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 µg/ml). (c) Blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 µg/ml), following protein precipitation with ACN; (d) blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), and NE (10 µg/ml), following protein precipitation with ACN with the supernatant spiked with 1-NIC (200 ng/ml); (e) a 2-h rat plasma sample obtained after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time t_R , including 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.4–5.9 min in different matrices).

in- and between-day precision values were 97–100 and 93–97%, respectively. Moreover, the protein precipitation with ACN for plasma samples resulted in the recovery of 1-NITC between 93 and 97% for both within- and between-day analysis.

At low (100 ng/ml), medium (5000 ng/ml), and high (50 000 ng/ml) concentrations of 1-NA, the within- and between-day accuracy was 96–106%, precision 97–99%, and recovery 95–110% (Table 3).

3.5. Stability

1-NITC was stable at temperatures of -20°C and -80°C in plasma, urine, ACN precipitated plasma and urine (Fig. 4a–d), and at all tested temperatures in ACN over 96 h (data not shown). However, 1-NITC degraded at RT and 4°C in plasma, urine, and ACN precipitated plasma and urine (Fig. 4a–d). The faster degradation at RT than at 4°C indicated a temperature-dependent pattern in each matrix (Fig.

4a–d). Moreover, the degradation of 1-NITC in plasma (Fig. 4a) and urine (Fig. 4c) was greater than that in ACN precipitated plasma (Fig. 4b) and urine (Fig. 4d) at same temperatures (RT and 4°C). The degradation of 1-NITC in ACN diluted urine (Fig. 4d) was much slower than ACN precipitated plasma (Fig. 4b); 1-NITC was stable when prepared in ACN at all temperatures (Fig. 4e). 1-NITC degraded with very similar patterns over the pH range of 2–10 over a 96-h period (Fig. 4f). A different pattern of degradation was observed at pH 11 (Fig. 4f); at pH 12 there was instantaneous degradation (data not shown). The degradation product of 1-NITC in universal buffer was confirmed to be 1-NA (data not shown). The degradation product of 1-NITC in plasma, urine, and ACN extracts of plasma and urine was not identified.

1-NA was stable in all matrices at RT, 4, -20 , and -80°C with quantitation variation less than 10% during individual test periods (plasma data only is shown in Fig. 4g); it was also stable over the pH

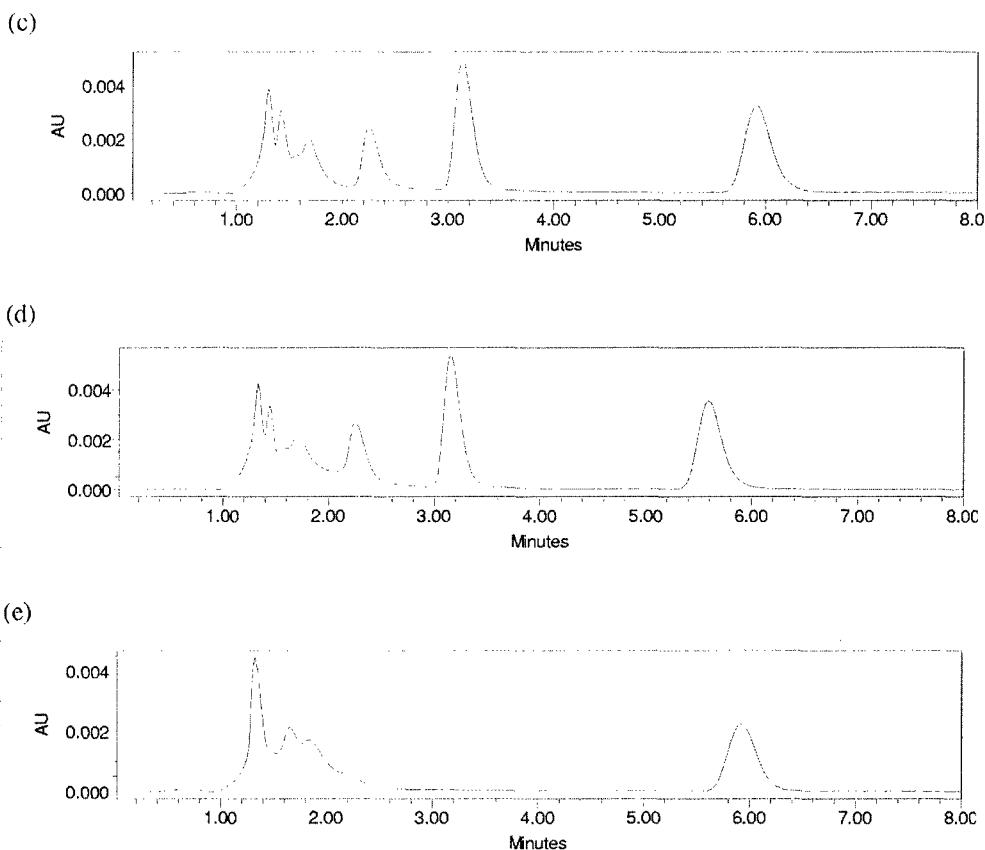


Fig. 2. (continued)

range of 2–12 (data not shown). In comparison, 1-NIC was stable when prepared in ACN (data not shown) but rapidly degraded in plasma (Fig. 2d), urine (Fig. 3c) and in ACN precipitated plasma and urine (data not shown). In universal buffer, 1-NIC was rapidly degraded to form 1-NA (data not shown).

3.6. Application of assay in rat pharmacokinetic studies

The described analytical method was used to analyze plasma and urine samples following the administration of 1-NITC (25 mg/kg i.v.) to a rat. The parent drug 1-NITC and metabolite 1-NA were the only compounds that could be detected in plasma and urine samples, respectively (Figs. 2e and 3d).

The concentration of 1-NITC in plasma over 24 h and 1-NA in urine over 25 h are given in Tables 4 and 5 and plasma data are plotted in Fig. 5. Using this HPLC assay, 1-NITC and 1-NA were quantified in rat plasma and urine, respectively (Tables 4 and 5). Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was about 0.4%.

4. Discussion

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination

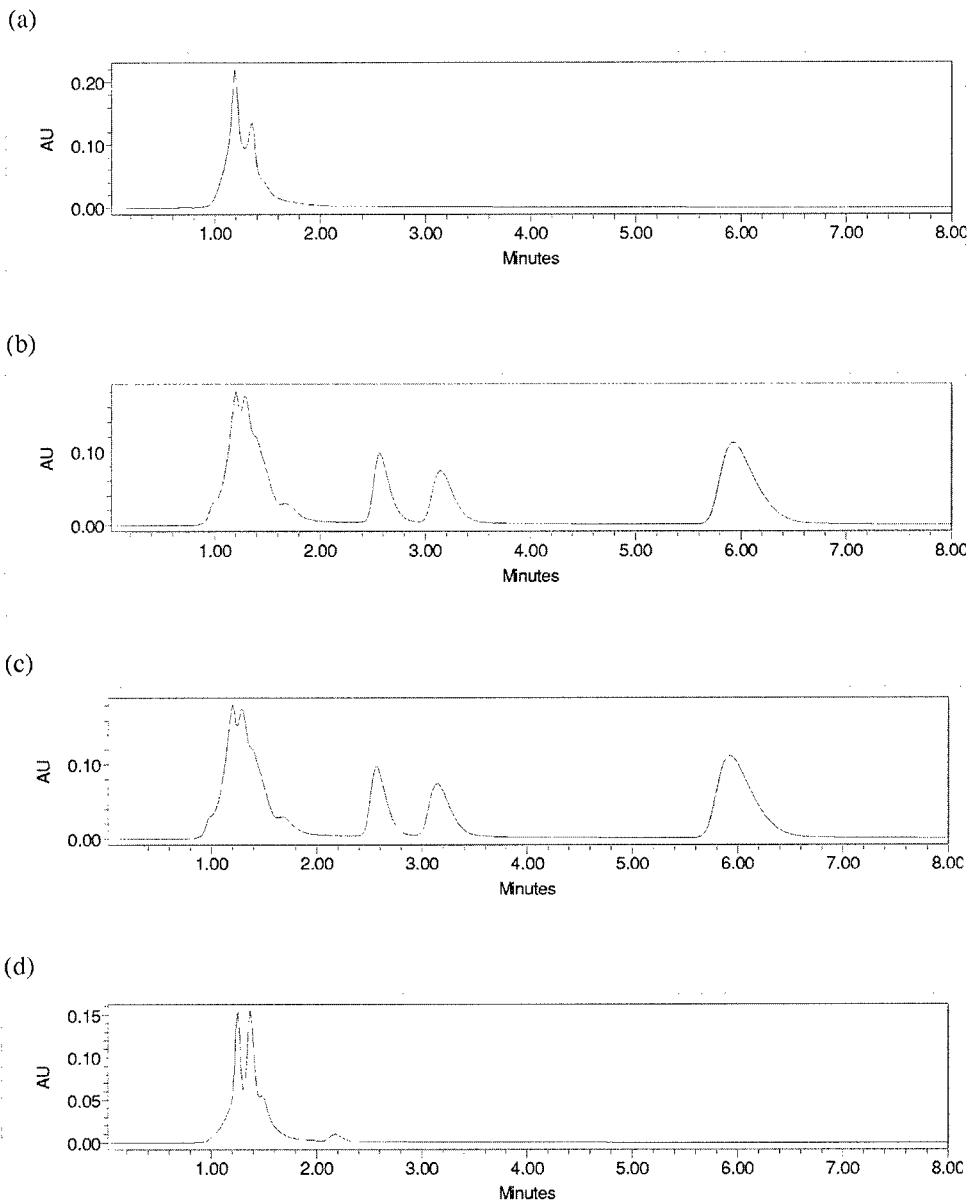


Fig. 3. Typical chromatograms for rat urine samples obtained from the analysis of (a) blank urine. (b) Blank urine with added 1-NITC (10 µg/ml), 1-NA (10 µg/ml), 1-NIC (10 µg/ml), and NE (40 µg/ml), followed by dilution with ACN; (c) blank urine with added 1-NITC (10 µg/ml), 1-NA (10 µg/ml), and NE (40 µg/ml), following dilution with ACN with the supernatant spiked with 1-NIC (10 µg/ml). (d) A urine sample obtained 2–4 h after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time t_R , including 1-NA (2.2–2.6 min), NE (3.2 min), and 1-NITC (6.0 min).

of α -naphthylisothiocyanate (1-NITC) and two metabolites α -naphthylisothiocyanate (1-NA) and α -naphthylisocyanate (1-NIC) in rat plasma and urine

has been developed. The features of the assay include the use of a reversed-phase column, UV detection, protein precipitation using ACN, and the

Table 1

The lower limit of quantitation of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine and ACN

| Compounds | LLQ in plasma (ng/ml) | LLQ in urine (ng/ml) | LLQ in ACN (ng/ml) |
|-----------|--------------------------|-------------------------|-----------------------|
| 1-NITC | 10 | 30 | 10 |
| 1-NA | 30 | 100 | 30 |
| 1-NIC | ND | ND | 30 |

ND: not detected in blank plasma and urine samples.

use of an internal standard. Through an extensive evaluation of the stabilities of 1-NITC and its metabolites in different biological matrices, we have optimized the conditions for the collection and storage of biological samples.

Based on the features of chemical structures (Fig. 1), naphthylene (NE) was selected as an ideal internal standard candidate. Additionally we found that other chemically unrelated compounds, such as acetophenone and propiophenone, could also be used as the internal standard in this assay. A single UV wavelength of 305 nm was used for the detection of 1-NITC, 1-NA, and 1-NIC in rat plasma and urine samples since we obtained the greatest sensitivity and minimal interference by endogenous compounds present in plasma and urine at this wavelength. Under the current HPLC conditions, the LLQ values were 0.5 ng (10 ng/ml) and 1.5 ng (30 ng/ml) for

1-NITC in plasma and urine, 1.5 ng (30 ng/ml) and 5 ng (100 ng/ml) for 1-NA in plasma and urine, respectively (Table 1).

The extraction of plasma samples was optimized by the use of a protein precipitation step with ACN at 4 °C. Using protein precipitation of plasma samples was more convenient and time-saving than liquid–liquid extraction and solid-phase extraction, and resulted in the least amount of interference with endogenous compounds, while retaining high extraction efficiency. Other organic solvents, such as methanol and acetone, were also investigated in our preliminary studies but produced endogenous interferences and/or variability in recovery. An extraction step for urine samples using ACN, methanol, acetone, and acetyl acetate (EtOAc) was also investigated, since the direct injection of urine supernatant resulted in tailing peaks of 1-NITC, 1-NA, and NE (data not shown). Extraction of urine samples with ACN at 4 °C resulted in the best accuracy, precision, and recovery.

The isothiocyanate group (N=C=S) in 1-NITC and the isocyanate group (N=C=O) in 1-NIC are highly reactive, undergoing hydrolysis. Therefore, the stabilities of 1-NITC, 1-NA, and 1-NIC were systematically investigated with regards to matrix and temperature effects over time. 1-NA was stable in all tested matrices at all tested temperature. However,

Table 2

The within- and between-day accuracy, precision, and recovery for 1-NITC in rat plasma

| | QC (ng/ml) | Accuracy (%) | Precision (%) | Recovery (%) |
|-------------|------------|--------------|---------------|--------------|
| Within-day | 10 | 106 | 97.6 | 93.2 |
| | 500 | 97.5 | 99.8 | 97.4 |
| | 5000 | 95.4 | 98.9 | 94.6 |
| Between-day | 10 | 102 | 92.9 | 95.9 |
| | 500 | 97.3 | 96.5 | 96.7 |
| | 5000 | 99.3 | 96.7 | 96.1 |

Table 3

The within- and between-day accuracy, precision, and recovery for 1-NA in rat urine

| | QC (ng/ml) | Accuracy (%) | Precision (%) | Recovery (%) |
|-------------|------------|--------------|---------------|--------------|
| Within-day | 100 | 106 | 98.7 | 102 |
| | 5000 | 98.2 | 99.4 | 107 |
| | 50 000 | 100 | 98.4 | 110 |
| Between-day | 100 | 105 | 97.4 | 95.4 |
| | 5000 | 96.3 | 97.7 | 104 |
| | 50 000 | 100 | 99.3 | 107 |

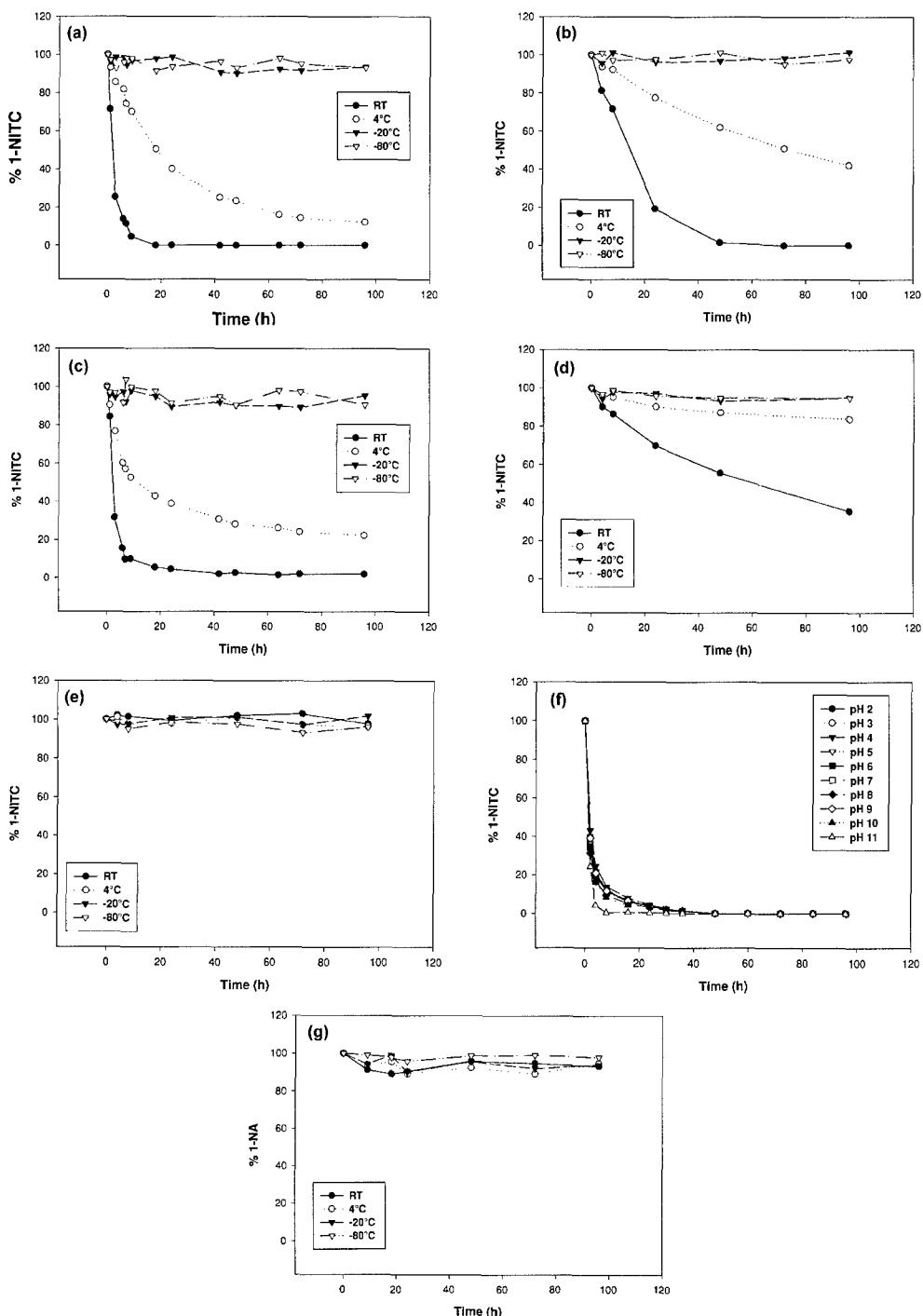


Fig. 4. The stability of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine, ACN precipitated plasma and urine, ACN, and universal buffer at RT, 4, -20, and -80 °C over 96 h. (a) The stability of 1-NITC in rat plasma at RT, 4, -20, and -80 °C. (b) The stability of 1-NITC in ACN precipitated rat plasma at RT, 4, -20, and -80 °C. (c) The stability of 1-NITC in rat urine at RT, 4, -20, and -80 °C. (d) The stability of 1-NITC in ACN diluted rat urine at RT, 4, -20, and -80 °C. (e) The stability of 1-NITC in ACN at RT, 4, -20, and -80 °C. (f) The stability of 1-NITC in universal buffer pH 2–11 at RT. (g) The stability of 1-NA in rat plasma at RT, 4, -20, and -80 °C.

Table 4
Concentrations of 1-NITC in rat plasma samples following a 25 mg/kg i.v. dose

| Time | Conc. (ng/ml) |
|--------|---------------|
| 5 min | 10 490±400 |
| 10 min | 7405±498 |
| 20 min | 4172±140 |
| 30 min | 3312±118 |
| 1 h | 1692±77 |
| 2 h | 1016±48 |
| 4 h | 702±36 |
| 6 h | 620±29 |
| 9 h | 351±15 |
| 12 h | 150±12 |
| 24 h | |

Data is mean±SD; n=3.

Table 5
Urinary excretion of 1-NA following a 25 mg/kg i.v. dose of 1-NITC to a female rat

| Time interval (h) | Vol. (ml) | Conc. (μ g/ml) | Amount (μ g) |
|-------------------|-----------|---------------------|-------------------|
| 0–2 | 8.2 | 0.42±0.04 | 3.44±0.33 |
| 2–4 | 3.8 | 2.02±0.23 | 7.68±0.87 |
| 4–6 | 1.5 | 2.64±0.35 | 3.96±0.52 |
| 6–9 | 1.5 | 2.17±0.25 | 3.26±0.37 |
| 9–24 | 30 | — | — |
| 24–25 | 3.2 | — | — |
| 0–25 | | | 18.34±2.09 |

Data is mean±SD, n=3. —: below detection limit.

the stabilities of 1-NITC and 1-NIC varied under different experimental conditions. The stability of 1-NITC was temperature-dependent in plasma, urine and ACN extracts of plasma and urine, i.e. stable at -20 and -80 °C but degraded at RT and 4 °C. Therefore, the plasma and urine samples obtained in our animal study were centrifuged at 4 °C and stored immediately at -80 °C. The standards of 1-NITC in plasma and urine for calibration curves and QCs were prepared individually on ice and assayed immediately at 4 °C using an autosampler. Under these conditions, the degradation of 1-NITC was less than 5% within 1 h for plasma samples and within 4 h for ACN extracts of plasma at 4 °C.

Our stability studies showed that 1-NITC and 1-NA were stable in plasma and urine at -80 °C when stored for more than 2 months (data not shown). The temperature-independent stability of 1-NITC in ACN indicated that ACN is an ideal extraction solvent for 1-NITC. In addition, the pH-independent degradation of 1-NITC in universal buffer further confirmed its high lability to hydrolysis. The degradation of 1-NITC at pH values of 2–10 was very similar to that of 1-NITC in plasma and urine samples at RT.

The isocyanate group was more reactive than the isothiocyanate group based on our study results. 1-NIC instantly degraded in aqueous matrix, i.e. plasma, urine, ACN precipitated plasma and urine,

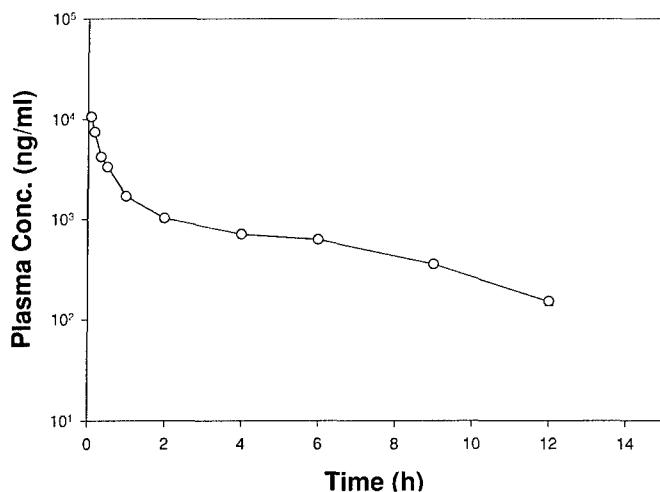


Fig. 5. Log plasma concentration vs. time relationship for 1-NITC after an i.v. bolus dose of 25 mg/kg.

and universal buffer. Although the degradation product of 1-NIC in plasma, urine, and ACN precipitated plasma and urine was not identified, the degradation product in universal buffer was confirmed to be 1-NA. In addition, the information on the stability in ACN indicated that 1-NIC (t_{R} 3.7 min) is stable in the mobile phase (ACN–H₂O 70:30, v/v) for at least 4 min, but probably shorter than 15 min (10 min for sample preparation and 5 min for mobile phase elution). Therefore the lack of detection of 1-NIC was probably due to its instability in the plasma and urine samples.

Using this HPLC assay, the concentrations of 1-NITC and 1-NA in rat plasma and urine, respectively, were determined (Tables 4 and 5). Our results agree with previous investigations demonstrating no unchanged 1-NITC in urine samples [12]. Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was low (0.4% of the injected dose of 1-NITC) indicating that 1-NITC and its metabolites may be eliminated by other mechanisms such as biliary excretion and CO₂ expiration, as reported by Capizzo and Roberts [11]. As well, there may be other unidentified metabolite(s) in urine rather than 1-NA.

5. Conclusion

In this paper, we have described a reversed-phase HPLC method for the quantitative determination of 1-NITC and metabolites 1-NA and 1-NIC in rat plasma and urine. The sample pretreatment procedure is based on a rapid precipitation step with ACN for both plasma and urine, thereby eliminating the need of laborious liquid–liquid extraction and solid-phase extraction techniques. The assay provides high sensitivity with LLQ values of 10, 30 and 10 ng/ml for 1-NITC in plasma, urine and ACN. The analysis method is precise and accurate, with the within- and between-day precision and accuracy within the range of 90–110% for QCs at low, medium and high concentration levels. The stability studies showed that 1-NITC was stable at all tested

temperatures in ACN, and at –20 and –80 °C in plasma, urine, and ACN extracts of plasma and urine, but degraded at RT and 4 °C. In universal buffer (pH 2–12) at RT, 1-NITC degraded with similar patterns at pH values ranging from 2 to 10; there was rapid degradation at pH 12. 1-NA was stable in all tested matrix at all temperatures (RT to –80 °C). 1-NIC was unstable with rapid degradation in plasma, urine, and ACN extracts of plasma and urine; however, 1-NIC was stable in ACN. The HPLC assay was successfully used in a preliminary rat pharmacokinetic study to analyze plasma and urine samples following the i.v. administration of 25 mg/kg 1-NITC.

Acknowledgements

This work was supported by grants from the Komen Breast Cancer Foundation and US Army Breast Cancer Research Program Contract DAMD17-00-1-0376. We acknowledge David M. Soda for his technical assistance.

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Determination of phenethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography–tandem mass spectrometry

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Received 26 May 2003

Abstract

Phenethyl isothiocyanate (PEITC) is a dietary compound present in cruciferous vegetables that has cancer-preventive properties. Our objective was to develop and validate a novel liquid chromatography–tandem mass spectrometry procedure to analyze PEITC concentrations in human plasma and urine. Following hexane extraction, ammonia was added to samples to derivatize PEITC to phenethylthiourea. Chromatographic separation was achieved on a C₁₈ column with acetonitrile/5 mM formic acid (60:40, v/v) as the mobile phase followed by tandem mass spectrometry detection in multiple reaction monitoring mode. Deuterium-labeled PEITC was used as the internal standard. The detection limit was 2 nM and calibration curves were linear from 7.8 to 2000 nM. The intra- and inter-day coefficients of variation were less than 5 and 10%, respectively. The intra- and inter-day accuracies ranged from 101.0 to 104.2% and from 102.8 to 118.6%, respectively. The recovery from spiked human plasma and urine ranged from 100.3 to 113.5% and from 98.3 to 103.9%, respectively. The assay was used to measure PEITC in plasma and urine samples obtained from subjects after consumption of 100 g of watercress. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentrations of PEITC.

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Keywords: PEITC; Phenethyl isothiocyanate; LC/MS/MS; Pharmacokinetics; Plasma; Urine

Organic isothiocyanates (ITCs)¹ are chemopreventive compounds occurring in a wide variety of cruciferous vegetables as glucosinolates. Damage to plant cells, such as from cutting and chewing, releases myrosinase which

catalyzes the hydrolysis of glucosinolates and the formation of ITCs by a Lossen rearrangement [1]. Phenethyl isothiocyanate (PEITC) (Fig. 1) is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low in vivo toxicity. Human exposure to PEITC is primarily through consumption of certain cruciferous vegetables containing its glucosinolate precursor, gluconasturtiin. Numerous cell and animal studies have demonstrated effective chemoprevention activity of PEITC for cancers, such as those of the lung, breast, esophagus, forestomach, pancreas, prostate, and colon, and with leukemia [2–4]. In addition, no toxicity has been observed in animal models with equivalent anticarcinogenic doses or at higher doses [1]. Based on promising animal studies of the efficacy and toxicity of PEITC, the compound is being studied with the long-term goal being development as a chemopreventive agent for lung cancer. A Phase I clinical trial

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¹ Abbreviations used: ITC, isothiocyanate; PEITC, phenethyl isothiocyanate; LC/MS/MS, liquid chromatography–tandem mass spectrometry; GC/MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; GC, gas chromatography; DTC, dithiocarbamate; PEITC-NAC, PEITC-N-acetylcysteine; MRM, multiple reaction monitoring; IS, internal standard; XIC, extracting ion current; LOD, limit of detection; LLOQ, lower limit of quantitation; k_a , absorption rate constant; Cl, clearance; V, volume of distribution; F, bioavailability; C_{\max} , maximal plasma concentration; t_{\max} , time to reach C_{\max} ; $t_{1/2}$, elimination half-life; Cl_R, renal clearance; A_e , amount excreted in the urine; AUC, area under the plasma concentration vs time curve; Q1, first quadruple; GST, glutathione S-transferase.

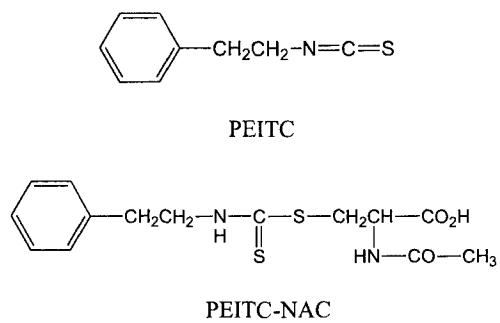


Fig. 1. Structures of PEITC and PEITC-NAC.

has been conducted by the National Cancer Institute to evaluate the safety and toxicity of PEITC in healthy individuals [5]. However, no study has specifically measured the concentration of unchanged PEITC in clinical samples. This information is necessary to evaluate *in vivo* concentration–effect and concentration–toxicity relationships and to design dosing regimens. Therefore, there is a need for an analytical method with the specificity and sensitivity to quantitate PEITC in human plasma samples.

Existing methods to analyze PEITC include high-performance liquid chromatography (HPLC), gas chromatography (GC) [6], gas chromatography–mass spectrometry (GC/MS) [7], and HPLC utilizing cyclocondensation derivatization [8–11]. When 40 mg of oral PEITC was administrated to humans, total ITC plasma concentrations were mainly within the nanomolar range [10]. HPLC and GC methods are not sensitive enough to analyze clinical samples containing this concentration of PEITC. GC/MS is able to measure individual ITCs [7]; however, the sensitivity of GC/MS is inadequate due to instrumental limitations and the volatility of PEITC which causes loss of the analyte during sample extraction. The HPLC-based cyclocondensation approach enhanced sensitivity significantly by derivatizing ITCs to 1,3-benzenedithiol-2-thione through a cyclocondensation reaction (Fig. 2A) and has been applied to analyzing plant materials, urine, and plasma. However, the assay lacks specificity for analyzing a particular ITC, since any ITC or dithiocarbamate (DTC) will form the identical product to be detected (Fig. 2A). Vegetables usually contain more than one ITC. Hence, if a vegetable such as watercress or broccoli sprouts is given to human subjects, the plasma profile of a specific ITC of interest (i.e., PEITC or sulforaphane) cannot be obtained. Even if a specific type of ITC is administered to subjects and food restriction is carried out before initiation of the study, there may still be trace amounts of other ITCs and DTCs present in the plasma due to the extensive exposure of humans to DTCs as fungicides, insecticides, pesticides, and rubber vulcanization accelerators [11] and to the complexity of human diets. Additionally, the major metabolite of PEITC, PEITC-

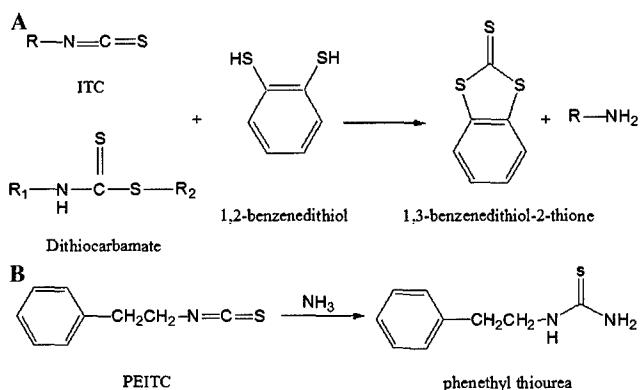


Fig. 2. Derivatization schemes for ITCs: (A) cyclocondensation reaction of ITCs and dithiocarbamates with 1,2-benzenedithiol to yield 1,3-benzenedithiol-2-thione; (B) reaction of PEITC with ammonia to yield phenethyl thiourea.

N-acetylcysteine (PEITC-NAC) (Fig. 1), is a DTC compound [12]. Therefore, when used to analyze plasma or urine concentrations of PEITC, the cyclocondensation approach would not be able to distinguish PEITC from its metabolites including PEITC-NAC, other ITCs, or DTCs.

In this study, we developed and validated a novel analytical approach that involves ammonia derivatization of PEITC to phenethylthiourea (Fig. 2B) and liquid chromatography–tandem mass spectrometry (LC/MS/MS) quantitation. The method is able to analyze PEITC in human plasma and urine selectively and accurately. The specificity and sensitivity were improved greatly over those of previous methods. We demonstrated the applicability of the method by analyzing PEITC in plasma samples obtained in a preliminary clinical pharmacokinetic study of PEITC in four healthy volunteers following watercress consumption.

Materials and methods

Materials

PEITC (99.9%), thiophosgene, ammonia (2 M in 2-propanol), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). 2-Phenylethyl-1,1,2,2-²H₄-amine (99.3% atom %D) was purchased from CDN isotopes (Quebec, Canada). PEITC-NAC was kindly provided by Dr. Fung-Lung Chung (American Health Foundation, Valhalla, NY). Watercress was purchased from a local grocery store (Wegmans, Buffalo, NY). All the solvents were HPLC grade and were purchased from Fisher Scientific (Springfield, NJ).

Synthesis of 1,1,2,2-²H₄-PEITC

1,1,2,2-²H₄-PEITC was synthesized from 2-phenylethyl-1,1,2,2-²H₄-amine and thiophosgene following

previously reported procedure [13]. The purity was >99% by HPLC and nondeuterated PEITC was not detected by mass spectrometry.

Preparation of standard solutions

A working stock solution of 25 μM PEITC in acetonitrile was prepared from a stock solution of 3 mM PEITC in acetonitrile. Calibration standards were prepared by appropriate dilution of PEITC to concentrations of 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500, and 2000 nM in HPLC water containing 300 nM of 1,1,2,2-H₄-PEITC as internal standard. Standard solutions for precision and accuracy determinations were prepared at PEITC concentrations of 20, 500, and 1500 nM in HPLC water. Recovery samples for human plasma and urine samples were prepared by adding the PEITC aqueous solutions to a plasma or urine sample at three levels (50, 500, and 1500 nM).

Clinical specimen collection and preparation

Four healthy volunteers (two Caucasians and two Asians), ages 21 years and older, were recruited from students and staff at the University at Buffalo. Informed consent was obtained from each subject. Subjects were not allowed to have taken any medications or any cruciferous vegetables (such as watercress, broccoli, cauliflower, brussel sprouts, radishes, turnips, or cabbage), condiments (such as horseradish, mustard, or soy sauce), or herbal products for 3 days prior to the study and did not eat or drink anything, except water, after midnight before the study day. Subjects ingested watercress at a dose of 100 g on the study day. Blood samples were collected using venipuncture with heparinized sterile glass tubes. Approximately 5 ml of blood was collected prior to the ingestion of watercress, and then repeated samples were collected at 7.5, 15, 30, and 45 min, and at 1, 1.5, 2, 3, 4, 6, 8, and 24 h after ingestion. A urine sample was collected one day prior to the study and on the study day at intervals of 0–1, 1–2, 2–4, 4–6, 6–8, 8–12, and 12–24 h. Subjects had free access to water, but not to food, until 3 h after the ingestion of watercress. No cruciferous vegetables or condiments (as listed above) were allowed during the study period.

Blood samples were collected into glass tubes containing heparin and centrifuged at 1500g for 10 min. The plasma was transferred into polyethylene tubes and kept frozen at -80 °C until analysis. Urine samples were collected into polyethylene tubes and kept frozen at -80 °C until analysis.

Sample extraction and derivatization

An aliquot of 0.5-ml of human plasma or urine sample was transferred into a 5 ml glass tube and

spiked with 6 μl of 25 μM (equivalent to 300 nM) IS; 1 ml *n*-hexane was added and the mixture gently vortexed for 20 s. After centrifugation at 1000g for 3 min, the hexane phase was removed and placed in a 10-ml capped glass screw-top tube. A second extraction was repeated; two hexane extracts were combined and 2 ml of ammonia (2 M in 2-propanol) was added for derivatization. The mixture was incubated on a shaking bed and allowed to react for 6 h at room temperature. The mixture was then dried under a N₂ stream at 50 °C and reconstituted with 50 μl of acetonitrile/H₂O (3:2, v/v) by vortex mixing. The reconstituted sample was transferred into a 200-μl autosampler vial insert for analysis by LC/MS/MS.

LC/MS/MS

The LC/MS/MS system consisted of a PE SCIEX API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a heated nebulizer interface, a Series 2000 Perkin-Elmer pump, and a Series 2000 Perkin-Elmer autosampler (Shelton, CT). HPLC separation was carried out on a C₁₈ (particle size 5 μm; 150 × 4.6 mm) column (Alltech, Deerfield, IL) and the mobile phase consisted of acetonitrile/5 mM formic acid (60:40, v/v). The flow rate was 1 ml/min and the injection volume was 5 μl. The mass spectrometer was operated in a positive ionization mode and the mass spectrometer setting was optimized for phenethylthiourea to give optimum ion yield. The pressure of the collision gas (N₂) was 2.8 mTorr, and the flow rates of the nebulizer gas (air) and curtain gas (N₂) were 1.2 and 0.8 L/min, respectively. The interface temperature was set at 350 °C. The declustering, focusing and entrance potentials were 20, 350, and -10 V, respectively. The nebulizer current was 3.0 μA and fragmentation was induced with a collision energy of 25 eV. Multiple reaction monitoring (MRM) of MS/MS was used for specific detection of the derivatives of PEITC and IS by measuring the characteristic ion transitions of *m/z* 181 (parent ion) to *m/z* 105 (product ion) and *m/z* 185 (parent ion) to *m/z* 109 (product ion), respectively.

Calibration and validation

Calibration was performed by an internal standard method. The integration was processed on Analyst software (Applied Biosystems, Foster City, CA), and calibration curves were obtained by plotting the extracting ion current (XIC) peak area ratios of analyte/internal standard vs concentrations. Standard curves were run on each analysis day and the coefficient of determination *r*² was used to judge linearity.

Intra- and interday precision and accuracy and recovery from plasma and urine samples were assessed

through triplicate analyses of the same samples containing known amounts of PEITC, with three samples per concentration level. Precision was estimated as CV% of the mean of all the determinations at each concentration level. Accuracy was determined by comparing the calculated concentrations to the known concentrations. Recovery was calculated by comparing the determined amounts for extracted blood or urine samples with the known amounts added. The limit of detection (LOD) was assessed as the PEITC concentration at a signal-to-noise of 3:1. The lower limit of quantitation (LLOQ) was defined as the PEITC concentration yielding a mean assayed concentration within 20% of the known concentration and a precision with a CV% less than 20%.

Pharmacokinetic analysis

The plasma concentrations over time were evaluated and the pharmacokinetic parameters were estimated by noncompartmental and compartmental model analysis using WinNonlin Professional Edition Version 2.1 (Pharsight, Mountainview, CA). The dose was estimated to be 25 mg PEITC, based on the calculation that 30 mg watercress contained 7.6 mg PEITC [14]. Apparent absorption rate constant (k_a), clearance (Cl/F), and volume of distribution (V/F) were calculated from the compartmental fit of the data. The maximal plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were determined directly from the plasma concentration vs time profile. The elimination half-life ($t_{1/2}$) was estimated from the terminal slope of the plasma concentration profile. Renal clearance (Cl_R) was calculated by dividing the total amount of PEITC excreted in the urine (A_e) by the area under the plasma concentration vs time curve (AUC).

Results and discussion

Selective extraction of PEITC from the matrix

The selective extraction of PEITC from the biological matrix is necessary to avoid interference from other components, especially PEITC metabolites. We separated PEITC from polar substances present in plasma or urine samples by extraction with hexane, a nonpolar solvent. PEITC-NAC, the major metabolite of PEITC, and other potential metabolites generated along the mercapturic acid pathway are polar compounds that would not be extracted into hexane. Analysis of the hexane phase after extraction of an aqueous solution of PEITC-NAC revealed that no PEITC-NAC was present (data not shown). Hexane extraction was conducted twice to ensure that the majority of the analyte was extracted taking account of the low amount in clinical

samples. In addition, increased extraction times did not show any significant improvement of the assay sensitivity.

Derivatization of PEITC to phenethylthiourea

After the hexane extraction, an excess amount of ammonia (in 2-propanol) was added to the organic phase. The reaction of PEITC and ammonia occurs efficiently at room temperature. LC/MS is of restricted usage if one is analyzing PEITC directly primarily due to the low ionization capability of the compound. On the other hand, ITCs are reactive electrophilic compounds that easily react with *O*-, *S*-, and *N*-nucleophiles. Due to good nucleophilicity of amines, we initially investigated the use of phenethylamine to react with PEITC. The UV absorptivity of the derivative was substantially higher than that of PEITC, and so HPLC could possibly be used to analyze the clinical samples. However, the excess amount of phenethylamine was hard to remove and the chromatographic peak of phenethylamine tailed and interfered with the peak of the derivative. Since volatile amines would not have this problem because of their easier removal, we chose to investigate the use of ammonia for the reaction with PEITC. We found that this reaction was highly efficient at room temperature in aqueous solution. However, PEITC was present in the hexane phase after extraction, resulting in an inefficient reaction with ammonia aqueous solution due to the immiscibility of the two phases. We found that 2-propanol mixed thoroughly with hexane; therefore, ammonia in 2-propanol solution was utilized and the reaction was complete within 6–8 h. The product, phenethylthiourea, is polar and nonvolatile; hence it represents a good substrate for LC/MS analysis, which was demonstrated by the mass spectra (Fig. 3). Additionally, the loss of PEITC during sample extraction and N₂ evaporation, due to its volatility, is avoided.

Unlike the cyclocondensation derivatization, the thiourea derivative is unique for each ITC (Fig. 2). Therefore, the interference from other ITCs was prevented by designation of the ions specifically from the PEITC derivative in MRM and by separation of the eluates by HPLC. It is highly unlikely that another compound would form a derivative that would have the same molecular and fragment ions and the same retention time as those of PEITC. Although PEITC-NAC and some other metabolites may also form phenethylthiourea after reaction with ammonia, they were removed during extraction and would not be present in hexane extracts for derivatization. Thus, the interference from PEITC metabolites was avoided. As a consequence, this analytical approach allowed the specific detection of PEITC with minimal interference from other ITCs, the PEITC metabolites and DTCs.

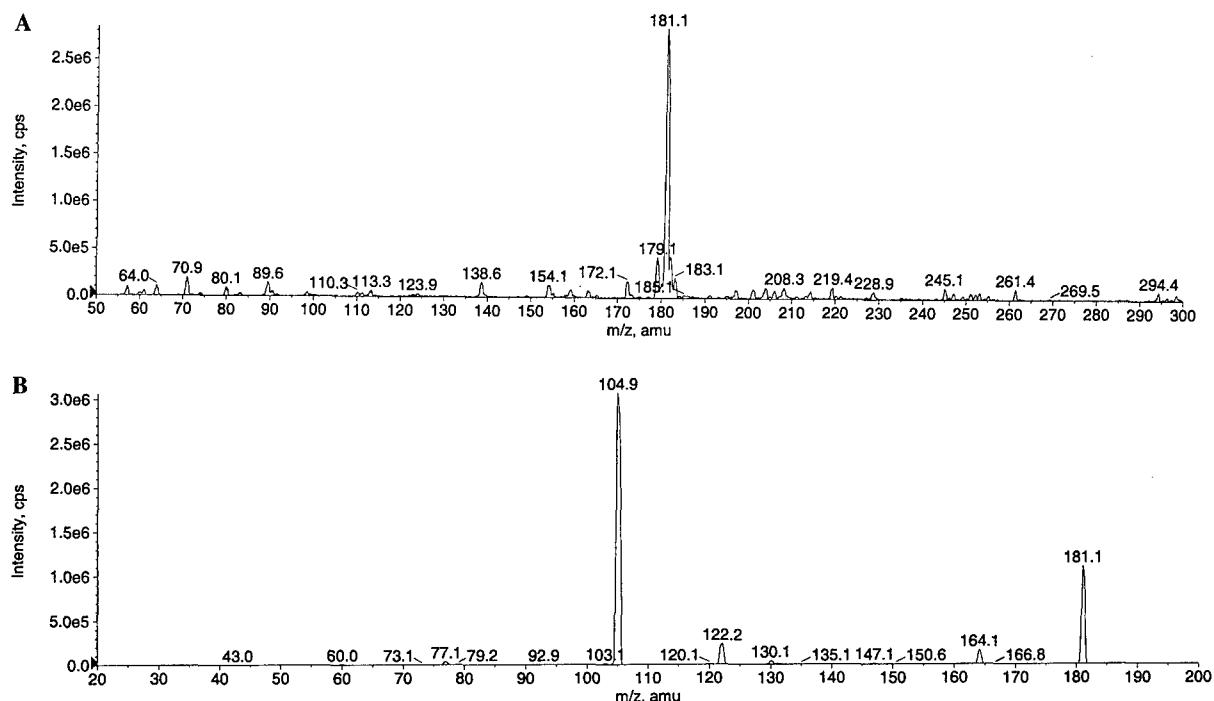


Fig. 3. Q1 full scan (A) and product ion scan (B) mass spectra of phenethylthiourea in standard solution. Phenethylthiourea was derivatized experimentally from PEITC standard after reaction with ammonia. The mass spectra were obtained by direct infusion of 1 $\mu\text{g}/\text{ml}$ of phenethylthiourea in acetonitrile/ H_2O (3:2, v/v) into the mass spectrometer.

LC/MS/MS

The Q1 (first quadrupole) full scan and product ion scan mass spectra of phenethylthiourea standard are presented in Figs. 3A and B, respectively. Molecular ion m/z 181 ($[\text{M}+\text{H}]^+$) was the most abundant ion (Fig. 3A). The most intensive product ion from the parent ion m/z 181 was m/z 105 (Fig. 3B) due to the loss of the thiourea fragment, which is a specific neutral loss for thiourea compounds. Therefore, the analyses were performed using MRM pairs of m/z 181 → 105 for the analyte.

The retention time of the thiourea derivative of PEITC was typically 2.2 min (Fig. 4). The noise level was low with the intensity lower than 200 counts per second (cps) and no interfering peaks at the retention time of 2.2 min were found on analysis of the plasma and urine samples from four subjects. Due to the high specificity of MS/MS, the thiourea derivative of PEITC was unambiguously identified by its MRM pairs (181 → 105), even if it coeluted with other derivatized ITCs.

Validation of the assay

Based on the PEITC level present in clinical samples, quantitation was typically performed at nanomolar concentrations. Validation was performed with regard to LOD, LLOQ, linearity, intraday and interday precisions and accuracies, and recoveries in human plasma

and urine. LOD and LLOQ are important characteristics for assay sensitivity when biological matrixes are analyzed, particularly when the analyte is present at low or trace concentrations. Based on six replicates assayed on three different occasions, the LOD was 2 nM and the LLOQ was 7.8 nM. In addition to much better selectivity, our method has improved sensitivity compared to the modified cyclocondensation method for clinical purposes (total ITC has LOD and LLOQ of 20 and 98 nM, respectively) [10].

Calibration curves of PEITC were linear over the concentration range of 7.8–2000 nM (r^2 values were typically greater than 0.995) (Fig. 5). During quantitative analysis, the variation in signal response due to external causes, such as contaminants in the ion source and ionization efficiency, are corrected by the internal standard that ionizes simultaneously with the analyte. Since the internal standard that we used is an isotopically labeled analogue, the ionization and fragmentation behavior are exactly identical to those of the analyte; therefore, we were able to obtain good linearity.

For precision, %CV values for PEITC were less than 5% for intraday analysis and less than 10% for interday analysis (Table 1). The intra- and interday percentage accuracies for PEITC were 101–105 and 102–119%, respectively (Table 1). The recoveries of PEITC from spiked human plasma and urine samples were within 14 and 4% of theoretical values (Table 2). These data

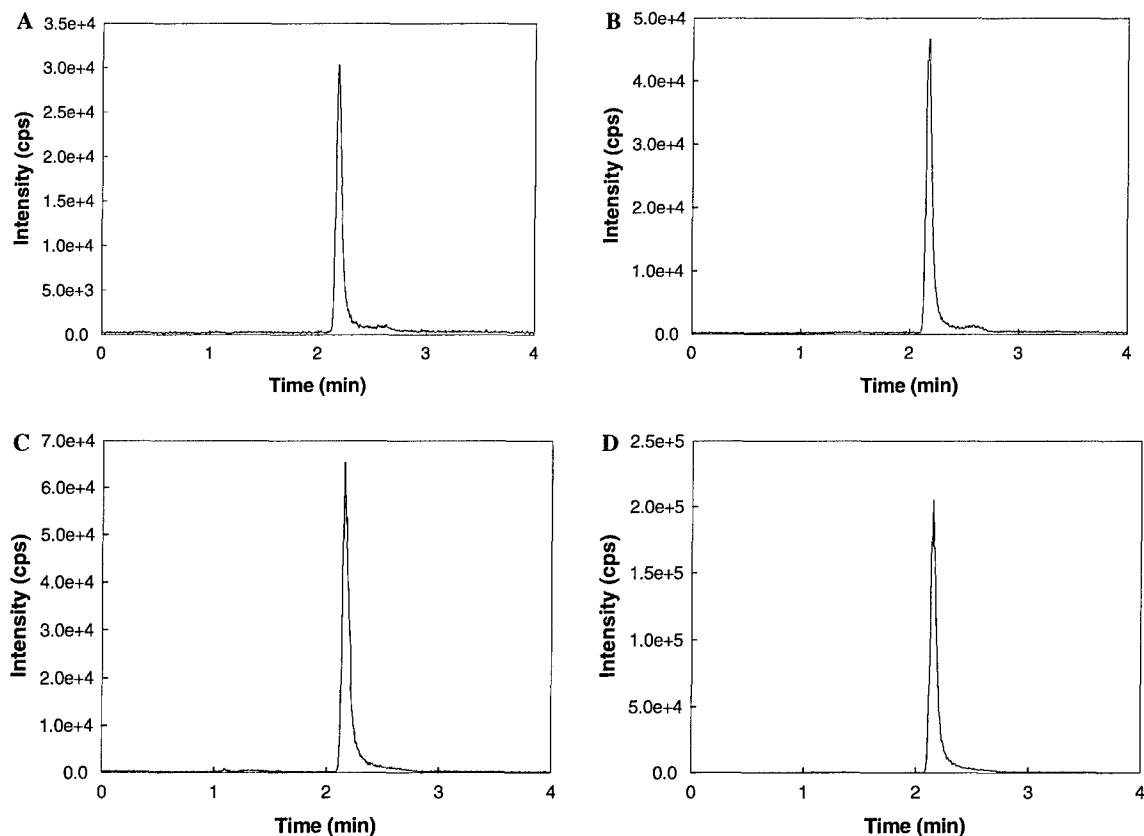


Fig. 4. Chromatograms of LC/MS/MS analyses of human plasma (obtained during a clinical study) containing analyte (A) and internal standard (spiked) (B) and of human urine (obtained during a clinical study) containing analyte (C) and internal standard (spiked) (D). The derivative of PEITC, phenethylthiourea, has a retention time of 2.2 min.

demonstrate that the interference from the metabolites, other ITCs or DTCs, was not detectable and that the method is capable of quantitating PEITC in plasma and urine accurately and specifically.

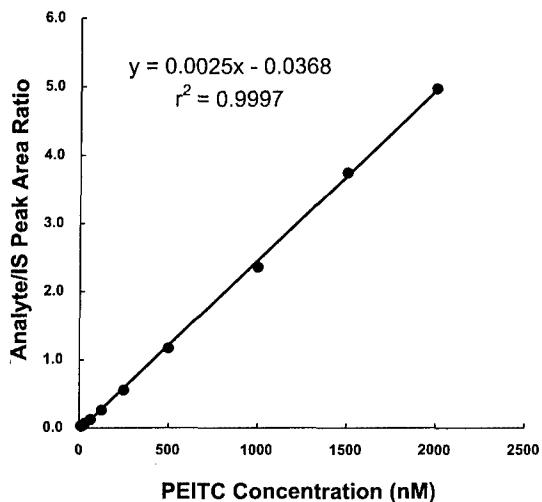


Fig. 5. Representative calibration curve for PEITC by LC/MS/MS analysis. Each point is the average of peak area ratios of duplicate injections for the derivative phenethylthiourea.

Clinical sample analysis

We detected and quantitated PEITC in most of the plasma and urine samples from four healthy volunteers after ingestion of 100 g watercress (approximately 25 g PEITC) and the plasma concentration vs time profile is shown in Fig. 6. It has been reported that little or no PEITC was detected in most of the plasma samples from volunteers who ingested 40 g of watercress (releasing 6–12 mg PEITC) and from subjects after 40 mg oral PEITC administration [10]. Additionally, PEITC was not detected in urine samples in a previous study of PEITC following watercress consumption using HPLC [14]. Therefore, our method showed improved sensitivity compared to those of previous studies.

Pharmacokinetics in humans after watercress consumption

The pharmacokinetic parameters of the four subjects are shown in Table 3. The plasma concentrations for the four subjects were fitted to a one-compartment model based on the criteria of goodness-of-fit (Fig. 6). However, due to our limited data collection between 8 and 24 h after watercress ingestion, we cannot

Table 1
Accuracy and precision of the LC/MS/MS assay of PEITC

| | Expected concentration (nM) | Mean measured concentration (nM) | SD | Precision (CV%) | Accuracy (%) |
|----------|-----------------------------|----------------------------------|------|-----------------|--------------|
| Intraday | 20 | 20.6 | 1.0 | 4.8 | 103.1 |
| | 500 | 505.0 | 6.5 | 1.3 | 101.0 |
| | 1500 | 1563.6 | 48.8 | 3.1 | 104.2 |
| Interday | 20 | 23.7 | 2.2 | 9.3 | 118.6 |
| | 500 | 533.4 | 31.1 | 5.8 | 106.7 |
| | 1500 | 1542.6 | 96.1 | 6.2 | 102.8 |

Table 2
Recovery of PEITC in human plasma and urine

| Concentration (nM) | Plasma | | | Urine | | |
|--------------------|--------------|-----|-----|--------------|-----|-----|
| | Recovery (%) | SD | CV% | Recovery (%) | SD | CV% |
| 50 | 113.5 | 5.2 | 4.6 | 98.3 | 8.7 | 8.8 |
| 500 | 100.3 | 2.1 | 2.1 | 103.9 | 3.4 | 3.3 |
| 1500 | 103.0 | 2.4 | 2.3 | 102.1 | 9.0 | 8.8 |

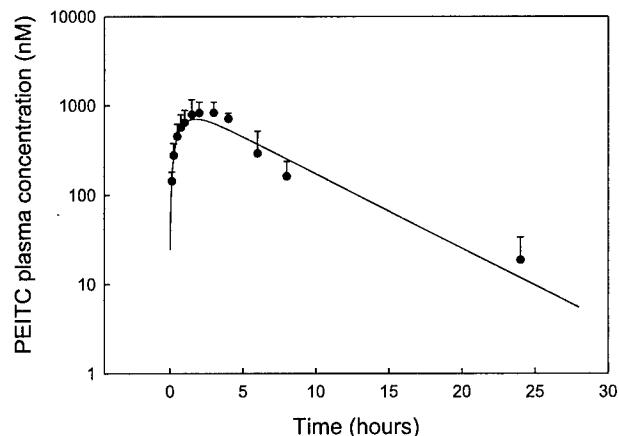


Fig. 6. Plasma concentration vs time profile of PEITC in humans following the consumption of 100 g watercress. Data are expressed as mean \pm SD, $n = 4$; closed circles represent the measured concentration and the line represents the predicted concentration fitted by WinNonlin.

preclude the possibility of fitting to a multicompartment model.

The mean endogenous plasma total ITC level in plasma samples obtained from subjects without food

restriction was $413 (\pm 193)$ nM (mean \pm SD), $n = 23$ [10]. When we analyzed plasma samples taken before the initiation of the study but after 3 days of dietary restriction, we still measured low concentrations of PEITC in the subjects (19.3 ± 14.3 nM, mean \pm SD, $n = 4$). Although we provided subjects with a list of restricted food items that are known to contain PEITC, it is likely that there are additional dietary sources of PEITC. Consequently, if human plasma is used as the matrix to perform calibration curves, baseline levels of PEITC need to be taken into account. Although the error may be minor when samples contain high concentrations, it would be significant for samples with low PEITC concentrations that are close to the baseline level. This may affect determination of some pharmacokinetic parameters. For example, elimination half-life is determined at later time points when PEITC concentrations are low and therefore may be biased by baseline levels of PEITC. Hence, we prepared different PEITC standards in water, to measure the absolute amount of PEITC in each sample, and then subtracted the baseline level from each sample measurement. The recovery from plasma and urine

Table 3
Pharmacokinetic parameters of PEITC in humans after ingestion of 100 g watercress

| Parameter | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Mean | SD | CV% |
|--------------------|-----------|-----------|-----------|-----------|-------|-------|------|
| C_{\max} (nM) | 753.4 | 1132.3 | 673.4 | 1155.0 | 928.5 | 250.7 | 27.0 |
| t_{\max} (h) | 2.0 | 3.0 | 4.0 | 1.5 | 2.6 | 1.1 | 42.2 |
| k_a (h $^{-1}$) | 1.5 | 1.0 | 1.0 | 1.5 | 1.3 | 0.3 | 22.1 |
| $t_{1/2}$ (h) | 5.9 | 5.7 | 3.6 | 4.4 | 4.9 | 1.1 | 22.8 |
| Cl/F (L/h) | 32.0 | 17.6 | 43.1 | 25.4 | 29.5 | 10.8 | 36.6 |
| V/F (L) | 193.8 | 110.4 | 196.1 | 117.6 | 154.5 | 46.8 | 30.3 |
| Cl_R (L/h) | | | 0.21 | 0.025 | | | |

C_{\max} , maximal plasma concentration; t_{\max} , time to reach C_{\max} ; k_a , absorption rate constant; $t_{1/2}$, elimination half-life; Cl , clearance; F , bioavailability; V , volume of distribution; Cl_R , renal clearance.

indicated that the assay is able to quantitate PEITC in those matrixes accurately.

The apparent absorption rate constant (k_a) determined for the four subjects was $1.3 (\pm 0.3) \text{ h}^{-1}$, based on a one-compartment model. This is a complex parameter because it represents not only the absorption rate but also the hydrolysis rate of gluconasturtiin to PEITC by the microflora present in the gastrointestinal tract. The t_{\max} value was $2.6 (\pm 1.1) \text{ h}$, indicating that plasma concentrations peak relatively rapidly. These data, taken together with the k_a value, suggest that the absorption of PEITC is relatively fast. Although PEITC has a low polarity and a low molecular weight and one might anticipate rapid absorption, we cannot exclude the possibility of flip-flop kinetics (absorption being slower than elimination) in this study. Total ITC (at least including PEITC and its conjugates) peaked at $4.6 (\pm 0.7) \text{ h}$ in three subjects after oral dosage of PEITC in capsules [10].

The elimination $t_{1/2}$ of PEITC in our study was $4.9 \pm 1.1 \text{ h}$, while total ITC had a $t_{1/2}$ of $3.7 \pm 1.3 \text{ h}$ [10]. In addition to the differences in dosage form and in chemical forms analyzed, the discrepancies can also result from interindividual variability and limited subject numbers. It has been known that glutathione S-transferase M1 (GSTM1) and GSTT1, the two major GSTs responsible for PEITC metabolism in humans, exhibit polymorphisms in the population due to homozygous deletion of the genes [15]. About 50% of the population have GSTM1 null type, while 12–16% of Germans and English and 60–64% of Chinese and Koreans have GSTT1 null type [16]. Consequently, presence or absence of the enzymes due to different genotypes can affect the metabolism greatly, resulting in variable $t_{1/2}$ and t_{\max} of PEITC, especially when the subject number is small.

Conclusion

A novel LC/MS/MS procedure with high sensitivity and specificity was developed and validated to analyze PEITC in human plasma and urine samples. The method consists of sample extraction by hexane, followed by its ammonia derivatization to thiourea, chromatographic separation on a C₁₈ column, and then detection in MRM mode. High selectivity was achieved by selective extraction of the analyte from the biological matrices by hexane, ammonia derivatization to thioureas that maintains the chemical identity of different ITCs, and the combination of HPLC with a specific MRM of a characteristic transition of the analyte derivative. The use of a stable isotopically labeled internal standard ensured the accuracy of quantitation and eliminated a matrix effect. To our knowledge, this is the first assay with the specificity and sensitivity to determine unchanged PEITC in biological samples.

Our preliminary clinical study demonstrated that the method was able to characterize the pharmacokinetics of PEITC in human subjects after watercress ingestion and therefore will be a valuable tool for further clinical investigations.

Acknowledgments

This work was funded by US Army Breast Cancer Research Program Contract DAMD17-00-1-0376. Y.J. is the recipient of a Pfizer Graduate Fellowship. We acknowledge Dr. Walter Conway for his insightful discussion, Mr. Tao Ji (Department of Chemistry, University at Buffalo) for his assistance in internal standard synthesis, and Ms. Lisa Predko for her assistance with the clinical study.

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Appendix 5

Effects of Benzyl-, Phenethyl-, and α -Naphthyl Isothiocyanates on P-glycoprotein- and MRP1-Mediated Transport

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Received 2 January 2004; revised 22 February 2004; accepted 11 March 2004

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20101

ABSTRACT: The objective of this investigation was to evaluate the effects of two dietary isothiocyanates (ITCs), benzyl- (BITC) and phenethyl isothiocyanate (PEITC), and one synthetic ITC, α -naphthyl isothiocyanate (1-NITC), on the P-glycoprotein (P-gp)- and multidrug-resistance protein 1 (MRP1)-mediated efflux of daunomycin (DNM), determine whether PEITC is a substrate of P-gp and/or MRP1, and elucidate the mechanism(s) involved in the inhibition of transport. BITC, PEITC, and 1-NITC significantly increased the 2-h accumulation of DNM in MCF-7/ADR (P-gp overexpression), PANC-1 (MRP1 overexpression), and human colon adenocarcinoma Caco-2 cells (except for 1-NITC). The accumulation of ^{14}C -PEITC was not changed in Caco-2, human breast cancer MDA435/LCC6 and MDA435/LCC6MDR1 (P-gp overexpression) cells in the absence and presence of the P-gp inhibitor verapamil, but significantly increased with the MRP inhibitor MK571 in PANC-1 cells. The isocyanate and amine metabolites had no effect on DNM accumulation in any cell line. After 2- and 24-h ITC treatments, cellular concentrations of glutathione (GSH) in PANC-1 and Caco-2 cells were depleted by BITC and PEITC, but not by 1-NITC; glutathione-S-transferase activity exhibited small changes. Our results suggest that (1) BITC, PEITC, and 1-NITC inhibit the P-gp- and MRP1-mediated efflux of DNM; (2) PEITC and/or its conjugates do not represent P-gp substrates; (3) BITC and PEITC, but not 1-NITC, inhibit MRP1 through the depletion of intracellular GSH, which acts as a cosubstrate for DNM efflux via MRP1; and (4) PEITC and/or its conjugates are MRP1 substrates so binding interactions with DNM represent a second potential mechanism involved in MRP1 inhibition. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:1901–1911, 2004

Keywords: benzyl isothiocyanate; phenethyl isothiocyanate; naphthyl isothiocyanate; daunomycin; P-glycoprotein; MRP1; multidrug resistance

INTRODUCTION

Inherent and/or acquired multidrug resistance (MDR) is a major obstacle to cancer chemotherapy. MDR has been attributed to the overexpression of different integral membrane proteins,

including the 170-kDa multidrug-resistance protein P-glycoprotein (P-gp)¹ and the 190-kDa multidrug-resistance protein 1 (MRP1).² These two extensively studied proteins belong to the ATP-binding cassette (ABC) transporters that use the energy of ATP hydrolysis to translocate their substrates across cellular membranes. P-gp and MRP1 transport a wide variety of structurally and functionally diverse but relatively lipophilic cancer chemotherapeutic agents. P-gp is thought to actively transport anticancer drugs in unmodified forms, including anthracyclines, vinca alkaloids,

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Journal of Pharmaceutical Sciences, Vol. 93, 1901–1911 (2004)
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epipodophyllotoxins, and taxanes. MRP1, the founding member of the nine-member MRP family, mediates the transport of chemotherapeutic drugs either unmodified or conjugated to anion ligands (e.g., glutathione, glucuronide, and sulfate) across cellular membranes. In fact, MRP1 shares many substrates with P-gp, although there is only 15% amino acid identity between the two transport proteins.²

Benzyl-(BITC) and phenethyl isothiocyanate (PEITC) (Fig. 1) belong to a group of naturally occurring organic isothiocyanates (ITCs; R-N=C=S) that are found abundantly in cruciferous vegetables, such as watercress, broccoli, cabbage, and cauliflower. BITC and PEITC are derived from their parent glucosinolates (β -thioglucoside N-hydroxysulfates) glucotropaeolin and gluconasturtin, respectively, following hydrolysis of the glucosinolates mediated by the enzyme myrosinase (β -thioglucosidase).³ These two natural products have attracted more and more attention as potent and selective inhibitors of carcinogenesis in various animal models,⁴ partly due to the direct inhibition and downregulation of the Phase I cytochrome P450 enzymes responsible for carcinogen activation,³ and induction of phase II enzymes such as glutathione S-transferases (GST) responsible for the detoxification of electrophilic intermediates.^{3,5} PEITC is capable of inducing apoptosis in a dose-dependant manner in HeLa cervical cancer cells, human leukemia HL-60 cells, PC-3 prostate cancer cells, and in human HT colon carcinoma cells.⁶ α -Naphthyl isothiocyanate (1-NITC) (Fig. 1), a synthetic ITC, was used as a carcinogenesis inhibitor in rats as early as the 1960s.⁴

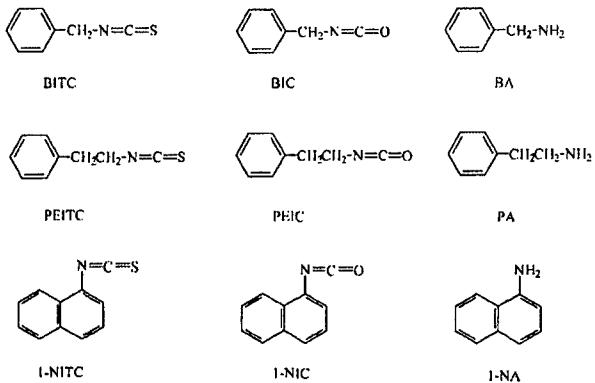


Figure 1. Chemical structures of BITC, PEITC, and 1-NITC and their corresponding isocyanates and amines.

Recently, our laboratory screened 12 organic ITCs and found that BITC, PEITC, and 1-NITC showed the greatest effects on the P-gp- and MRP1-mediated efflux of the anticancer drugs daunomycin and vinblastine in the human breast cancer cell line MCF-7/ADR (P-gp overexpression) and the pancreatic adenocarcinoma cell line PANC-1 (MRP1 overexpression).⁷ The objective of the present study was to confirm the ITC results in other cell lines examining the effects of the ITCs and their isocyanate and amine metabolites, determine whether PEITC represents a substrate of P-gp and/or MRP1, and to evaluate the possible mechanism(s) involved in the inhibition of transport of DNM by PEITC, BITC, and 1-NITC.

MATERIALS AND METHODS

Chemicals

Benzyl amine (BA), α -naphthyl amine (1-NA), α -naphthyl isocyanate (1-NIC), 1-NITC, phenethyl amine (PA), and verapamil (VRP) were purchased from Sigma (St. Louis, MO). Benzyl isocyanate (BIC), BITC, phenethyl isocyanate (PEIC), and PEITC were purchased from Aldrich (Milwaukee, WI). Chemical structures of tested compounds are shown as in Figure 1. DNM was purchased from Fluka (Switzerland) and ³H-DNM (18.5 Ci/mmol) from Perkin-Elmer (Boston, MA). ¹⁴C-PEITC (8.1 mCi/mmol) was synthesized by Qi Wang in our laboratory, with a radiochemical purity, based on HPLC, of 99.3%.^{8,9} MK571 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Acetonitrile was HPLC grade from Fisher Scientific (Fair Lawn, NJ). Other chemicals were analytical grade unless specified.

Cell Culture

Human breast cancer MCF-7 sensitive and MCF-7/ADR resistant cells were obtained from the National Cancer Institute, MDA435/LCC6 sensitive and MDA435/LCC6MDR1 resistant cells were generously provided by Dr. Robert Clarke (Lombardi Cancer Center, Georgetown University, Washington DC). Human colonic adenocarcinoma Caco-2 cells were a gift from Dr. Amrita Kamath (Bristol Myers Squibb, Princeton, NJ). Human pancreatic adenocarcinoma PANC-1 cells were obtained from American Type Culture Collection (Manassas, VA).

MCF-7 and MDA435/LCC6 cells (both wild type and MDR subtype) were cultured in 75 cm² flasks with RPMI 1640 culture media (GIBCO BRL, Buffalo, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO), Caco-2 cells with Eagle's minimum essential medium (GIBCO) supplemented with 10% FBS, 1% Eagle's minimum essential medium nonessential amino acids solution (10 mM) (GIBCO), and 1% antibiotic-antimycotic solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/ml amphotericin B), at 37°C in a humidified atmosphere with 5% CO₂/95% air. For MCF-7 and MCF-7/ADR, the culture media contained 100 U/mL penicillin and 100 µg/mL streptomycin. PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% FBS and 2.2 g/L sodium bicarbonate at 37°C in a humidified atmosphere with 10% CO₂/90% air. MCF-7 (both wild type and MDR subtype) cells with passage numbers of 16–24, MDA435/LCC6 (both wild type and MDR subtype) cells with passage numbers of 16–25, Caco-2 cells with passage numbers of 86–100, and PANC-1 cells with passage numbers of 64–74, were used for the experiments.

Accumulation Studies

Cells were seeded onto Falcon 35 × 10-mm dishes (Becton Dickinson) at a density of 5 × 10⁵ cells per dish and reached confluence after 2–3 days, except for the Caco-2 cells that were cultured for 21–25 days.¹⁰ Growth medium was removed and the cells were washed twice with sodium buffer (137 mM NaCl, 54 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, pH 7.4 adjusted by Tris base). For ³H-DNM accumulation studies, 1.0 mL of incubation buffer containing 0.05 µM ³H-DNM, 100 µM test drugs, or vehicle (0.2% DMSO used as control) was added. Verapamil (100 µM) was used as a positive control. For the ¹⁴C-PEITC accumulation studies, 1.0 mL of incubation buffer containing PEITC (1, 5, 20, 50, or 100 µM), with or without verapamil (100 µM) or MK-571 (100 µM) was added. The incubation was stopped at 2 h by rinsing the cells three times with ice-cold stop buffer (137 mM NaCl, 14 mM Tris, pH 7.4 adjusted by concentrated HCl). Cells were dissolved by adding 1.0 mL of a solution of 0.3 N NaOH and 1% sodium dodecylsulfate for 1 h, and 150-µL aliquots were used to determine radioactivity by liquid scintillation counting (Tri-Carb Liquid Scintillation Analyzer 1900 CA, Packard Instrument Co., Downers Grove, IL). The data

were normalized by the protein concentration in each dish using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). The accumulation level of ³H-DNM was expressed as a percentage of the control group. The accumulation level of ¹⁴C-PEITC was expressed as nmol PEITC per mg protein. For each sample, two or three separate experiments were performed, with triplicate determinations in each experiment.

GSH Assay

Cellular GSH concentrations were assayed by an enzymatic recycling procedure in which GSH is sequentially oxidized by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by β-NADPH in the presence of glutathione reductase (GR).¹¹ In brief, confluent PANC-1 or Caco-2 cells grown in Falcon 35 × 10-mm dishes were washed with PBS twice, and the ITCs (100 µM) were added in protein-free media for 2 or 24 h. After incubation, the cells were washed three times with ice-cold PBS and detached by scraping in 0.6 mL of 5% (w/v) sulfosalicylic acid (Sigma). Cells were then lysed by sonication (Branson Sonifier® Cell Disruptor 200) for 1 min and centrifuged at 10,000×g (Eppendorf Centrifuge 5417R, Brinkmann Instruments Inc., Westbury, NY) at 4°C for 10 min. The supernatant was used to determine the GSH concentration. Using 96-well microplates, 20 µL of samples or GSH standards, 10 µL of 3 mM DTNB (Sigma), and 70 µL of 0.3 mM β-NADPH (Sigma), were added into the wells. Upon the addition of GR (2.1 U/mL per well) (675 U/mL stock solution, from Baker yeast, ICN Biomedicals Inc., Aurora, OH), the enzyme kinetics were determined using a microplate spectrometer Spectra Max 340PC (Device Molecular, Sunnyvale, CA) at 405 nm and 25°C for 3 min at 0.5-min intervals. The GSH concentrations of the samples were calculated by the calibration curve plotted as δOD/min versus GSH standard concentration, normalized by protein concentrations in each dish measured by the BioRad protein assay kit, and expressed as percentages of the control values. Control experiments examined the potential interference of the test drugs on known concentrations of GSH without the presence of GR, and no effects were observed (data not shown).

GST Assay

Cellular GST activity was analyzed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate,

according to the method of Habig and Jakoby.¹² Briefly, PANC-1 cells were grown in 75 cm² flasks until confluent. The test drugs (100 µM) were added to each flask for 2- or 24-h periods. After incubation, cells were washed twice with ice-cold PBS, detached by scraping with 6 mL of 1 mM EDTA in PBS, and centrifuged at 5000×g (Sorvall RC-5B Refrigerated Superspeed Centrifuge). Cells were lysed by sonication in a 5 mM K₂HPO₄ buffer (pH 6.5 adjusted with concentrated H₃PO₄) and ultracentrifuged at 140,000×g (17 Ultracentrifuge, Beckman, Fullerton, CA) at 4°C for 45 min. GST activity was determined by measuring the conjugation of CDNB with GSH, using a microplate spectrometer at 340 nm and 25°C for 5 min at 0.5-min intervals. A unit of enzymatic activity was defined as the formation of 1 µmol of product per min at 25°C. The data was normalized by protein concentrations in each flask measured by the BioRad protein assay kit. The GST activity of samples was expressed as a percentage of the control value.

Stability of ITCs in Sodium Buffer

The stability of BITC, PEITC, and 1-NITC in sodium buffer were determined in both Falcon 35 × 10-mm polystyrene tissue culture dishes and 4-mL glass vials. Eight microliters (25 mM in DMSO) was added to 2.0 mL sodium buffer (pH 7.4) to obtain the final concentration of 100 µM, which was used in the accumulation studies. Three replicates of each sample were assayed at 2, 24, and 48 h using HPLC assays. The Waters HPLC system (Milford, MA) consisted of a 1525 binary pump, a 717plus autosampler configured with a heater/cooler, a Model 5HC column oven, and a 2487 UV detector. The column and autosampler temperatures were kept at room temperature (21 ± 1°C). The reversed-phased chromatography was performed with a stain-steel less Whatmann (Clifton, NJ) Partisphere C-18 5-µm column 125 × 4.6 mm i.d. protected by a guard column, and compounds were eluted isocratically with a mobile phase consisting of acetonitrile:H₂O at 70:30 (v/v) for 1-NITC, 1-NIC, and 1-NA,¹³ 60:40 (v/v) for BITC and PEITC, and 50:50 (v/v) for BIC, BA, PEIC, and PA. The flow rate was 1.0 mL/min and injection volume was 50 µL. The UV detector was set at the dual wavelengths of 254 and 305 nm. The retention times of BITC, BIC, BA, PEITC, PEIC, PA, 1-NITC, 1-NIC, and 1-NA were 4.0, 5.0, 2.2, 4.2, 5.7, 2.4, 5.8, 3.7, and 2.2 min, respectively. The Breeze System

software version 3.2 (Waters) was used for instrument control and data analysis.

Statistical Analysis

Statistical significance was determined by one-way ANOVA (Prism 3.0 software, GraphPad, San Diego, CA) followed by a Dunnett's post hoc test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Stability of ITCs in Sodium Buffer

In this study, the ITC stability in the sodium buffer used in our experiments (pH 7.4) was examined in Falcon 35 × 10-mm dishes over a period of 48 h (Fig. 2). ITC concentrations decreased in a bi-exponential manner, with rapid loss of ITC occurring over the first 2 h. In contrast, the loss of PEITC, BITC, and NITC between 2–48 h was much slower than during the first 2 h, and each plot roughly paralleled one another. At the end of the initial 2-h incubation, BITC, PEITC, and 1-NITC concentrations represented 59.2 ± 1.7%, 69.1 ± 1.6%, and 32.1 ± 1.9% of the initial concentrations. At 24 and 48 h, 42.9 ± 4.8% and 21.5 ± 0.1% for BITC, 53.8 ± 3.8% and 41.1 ± 2.9% for PEITC, 16.0 ± 1.1% and 9.4 ± 0.7% for 1-NITC remained, respectively. Following removal of the sodium buffer from the Falcon 35 × 10-mm dishes after the 24-h incubation and the addition of 2.0 mL of DMSO, we found that almost all the parent ITCs could be recovered in the DMSO,

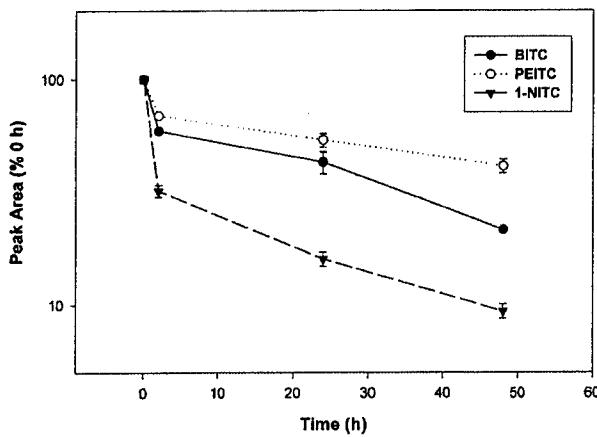


Figure 2. Loss of ITCs (100 µM) in sodium buffer in Falcon polystyrene 35 × 10-mm dishes ($n = 3$).

suggesting loss occurred predominantly due to adsorption to the plastic.

To further verify whether the loss of parent ITCs in sodium buffer within 2 and 24 h is due to hydrolysis or nonspecific binding to the dishes, we further investigated the stability of ITCs in 4-mL glass vials. After 2 h, $95.8 \pm 2.3\%$, $101.9 \pm 1.4\%$, and $85.0 \pm 5.4\%$ of the added BITC, PEITC, and 1-NITC, respectively, were present in the buffer, as detected by HPLC analysis. 1-NA, the degradation product of 1-NITC, was not detected. Similar recoveries were found after the 24-h incubations periods. We concluded that these three ITCs were stable in sodium buffer, and that the loss of parent ITCs was mainly due to nonspecific binding to plastics. Additional studies evaluated the stability of the metabolites in buffer solutions in Falcon 35 × 10-mm dishes. The metabolites BIC, PEIC, and 1-NIC degraded in buffer to form the corresponding amines (data not shown). BA, PA, and 1-NA were found to be stable in the buffer solutions (data not shown).

DNM Accumulation

The expression of P-gp and MRP1 has been evaluated by Western blot analysis to characterize the expression of these transport proteins in all cell lines used in this investigation. There was no detectable P-gp present in MCF-7 and PANC-1 cells, low expression of P-gp in MDA435/LCC6 cells, high P-gp in MCF-7/ADR, MDA435/

LCC6MDR1, and Caco-2 cells, and MRP1 expression in PANC-1 cells.^{10,14,15}

The time course for the uptake of DNM (0.05 μM) in the presence and absence of verapamil (100 μM) has been examined in MCF-7 and MCF-7/ADR cells in previous studies.⁷ It was found that equilibrium was achieved by 2 h in both cell lines. In our preliminary studies, we found that the uptake of DNM reached a plateau by 2 h in MDA435/LCC6, MDA435/LCC6MDR1, Caco-2, and PANC-1 cells as well (data not shown).

The accumulation of DNM was not changed in MCF-7 cells (no detectable P-gp expression), in the presence and absence of the test compounds and verapamil (Table 1). For MCF-7/ADR cells (P-gp overexpression), the accumulation of DNM was significantly increased in the presence of BITC ($209 \pm 24.7\%$; $p < 0.001$), PEITC ($198 \pm 63.1\%$; $p < 0.001$), and 1-NITC ($487 \pm 26.7\%$; $p < 0.001$), with verapamil ($347 \pm 65.2\%$; $p < 0.001$) as the positive control (Table 1). BIC, PEIC, 1-NIC, BA, PA, and 1-NA did not increase the cellular accumulation of DNM.

For Caco-2 monolayers (Table 1), BITC ($175 \pm 50.4\%$; $p < 0.001$), PEITC ($185 \pm 21.3\%$; $p < 0.001$) and verapamil ($266 \pm 25.1\%$; $p < 0.001$) exhibited very similar effects as those observed in MCF-7/ADR cells. 1-NITC did not show any effect on the accumulation of DNM in this cell line. Similarly, BIC, PEIC, 1-NIC, BA, PA, and 1-NA did not have any effect on DNM accumulation in Caco-2 cells.

Table 1. The Effects of Isothiocyanates, Isocyanates, and Amines (100 μM) on the Accumulation of DNM (0.05 μM) in MCF-7, MCF-7/ADR, Caco-2, and PANC-1 Cells

| Com- ounds | Cell Lines | | | |
|---------------|-------------|-------------------------|-------------------------|-------------------------|
| | MCF-7 | MCF-7/ADR | Caco-2 | PANC-1 |
| Control | 100 ± 22.9 | 100 ± 2.3 | 100 ± 5.7 | 100 ± 4.0 |
| VRP | 155 ± 14.9 | 347 ± 65.2 ^a | 266 ± 25.1 ^a | NT |
| MK-571 | NT | NT | NT | 245 ± 17.1 ^a |
| BITC | 116 ± 9.8 | 209 ± 24.7 ^a | 175 ± 50.4 ^a | 134 ± 4.9 ^a |
| PEITC | 119 ± 2.3 | 198 ± 63.1 ^a | 185 ± 21.3 ^a | 141 ± 16.2 ^a |
| 1-NITC | 80.1 ± 12.2 | 487 ± 26.7 ^a | 103 ± 24.1 | 256 ± 3.7 ^a |
| BIC | 93.7 ± 12.2 | 109 ± 8.8 | 96.4 ± 8.0 | 116 ± 6.8 |
| PEIC | 103 ± 8.9 | 110 ± 5.6 | 83.5 ± 10.5 | 100 ± 5.6 |
| 1-NIC | 89.4 ± 10.3 | 94.2 ± 7.1 | 95.8 ± 4.2 | 90.8 ± 6.5 |
| BA | 85.1 ± 10.4 | 90.3 ± 8.0 | 103 ± 6.4 | 106 ± 11.4 |
| PA | 68.1 ± 30.3 | 97.9 ± 13.7 | 87.6 ± 12.0 | 92.9 ± 5.0 |
| 1-NA | 83.9 ± 16.7 | 72.3 ± 16.7 | 104 ± 15.3 | 87.3 ± 10.4 |

^aThe data are expressed as mean ± SD, $n = 6$, $p < 0.001$.

Verapamil (VRP) and MK-571 represent positive controls.

NT: not tested.

For PANC-1 cells (MRP1 overexpression), BITC ($134 \pm 4.9\%$; $p < 0.001$) and PEITC ($141 \pm 16.2\%$; $p < 0.001$) showed relatively small, but statistically significant effects on the accumulation of DNM (Table 1). The positive control, MK571 ($245 \pm 17.1\%$; $p < 0.001$) and 1-NITC ($256 \pm 3.7\%$; $p < 0.001$) exhibited greater effects on DNM accumulation. The isocyanate and amine metabolites did not alter DNM accumulation.

PEITC Accumulation

To examine whether ITCs are substrates of P-gp and/or MRP1, ^{14}C -PEITC was used in accumulation studies in the presence and absence of P-gp and MRP1 inhibitors. As shown in Figures 3 and 4, the accumulation of PEITC was dose dependent in the presence and absence of verapamil or MK571 in Caco-2, MDA435/LCC6, MDA435/LCC6MDR1, and PANC-1 cells. However, the addition of verapamil did not significantly increase the accumulation of PEITC in Caco-2 (Fig. 3A) and MDA435/LCC6MDR1 cell lines (Fig. 3B), indicating that PEITC is not a P-gp substrate. Verapamil can significantly increase the accumulation of the P-gp substrate DNM in Caco-2 cells (Table 1) and in MDA435/LCC6MDR1 cells.¹⁴ Additionally, the intracellular concentrations of PEITC were similar in MDA435/LCC6 and MDA435/LCC6MDR1 cells. If PEITC represents a substrate for P-gp, one would expect lower intracellular concentrations in the P-gp-overexpressing cell line MDA435/LCC6MDR1. In PANC-1 cells, MK571 significantly increased the uptake of PEITC at low

concentrations ($1 \mu\text{M}$, 6.8-fold, $5 \mu\text{M}$, 5.9-fold, and $20 \mu\text{M}$, 1.9-fold; $p < 0.001$), suggesting that PEITC or a metabolite of PEITC that retains the ^{14}C label is a MRP1 substrate (Fig. 4). However, MK571 did not change ^{14}C -PEITC accumulation at higher concentrations (50 and $100 \mu\text{M}$), indicating the possibility that there may be saturation of MRP1-mediated transport.

GSH Assay

The cellular GSH concentrations in PANC-1 and Caco-2 cells were determined after incubation with ITCs or their metabolites ($100 \mu\text{M}$) for 2 and 24 h. In this study, we found that the cellular GSH concentrations ranged from 10 to 30 mM in PANC-1 and Caco-2 cells (data not shown). As shown in Figure 5, BITC and PEITC significantly decreased the cellular GSH concentrations after both a 2-h incubation ($15.3 \pm 6.4\%$ of control value for BITC and $10.4 \pm 3.5\%$ for PEITC; $p < 0.001$) and a 24-h incubation ($1.0 \pm 0.6\%$ of control value for BITC and $2.3 \pm 0.4\%$ for PEITC; $p < 0.001$). However, 1-NITC did not change cellular GSH concentration at either time point ($90.4 \pm 16.7\%$ of control value after 2 h and $108 \pm 18.6\%$ after 24 h). Moreover, the effect of BITC and PEITC on cellular GSH was concentration dependent as determined after 2-h incubations, demonstrating significant effects at $5-10 \mu\text{M}$ concentrations (Fig. 6). On the other hand, no effect was observed for 1-NITC over a concentration range from 1 to $100 \mu\text{M}$ (Fig. 6). Similar effects of BITC and PEITC on cellular GSH concentration were also observed in Caco-2 cells (Fig. 7). After a 2-h

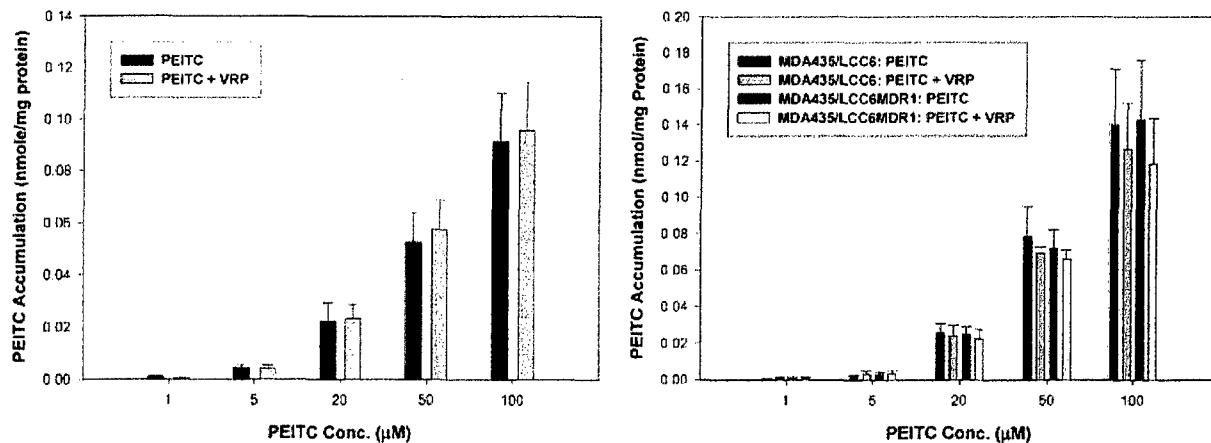


Figure 3. The effect of verapamil (VRP) ($100 \mu\text{M}$) on the accumulation of PEITC in (A) Caco-2 cells, (B) MDA435/LCC6, and MDA435/LCC6MDR1 cells. The data are expressed as mean \pm SD, $n = 6$.

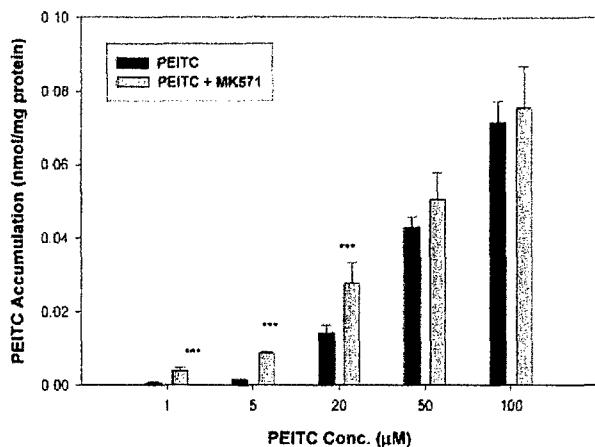


Figure 4. The effect of MK571 (100 μ M) on the accumulation of PEITC in PANC-1 cells. The data are expressed as mean \pm SD, $n = 6$, *** $p < 0.001$.

incubation, the cellular GSH concentrations were $5.7 \pm 0.2\%$ ($p < 0.001$) and $8.2 \pm 6.2\%$ ($p < 0.001$) of the control value for BITC and PEITC (100 μ M) treatment, respectively. 1-NITC did not alter GSH concentrations ($93.1 \pm 2.6\%$ of control value) in Caco-2 cells.

GST Assay

The cellular GST activity in PANC-1 cells was investigated in the presence of ITCs (100 μ M) following 2- and 24-h incubations (Fig. 8). PEITC ($78.6 \pm 4.6\%$ of control value; $p < 0.001$) and 1-NITC ($84.7 \pm 4.3\%$; $p < 0.001$) produced small but significant decreases in GST activity after a 2-h treatment, and small but significant increases

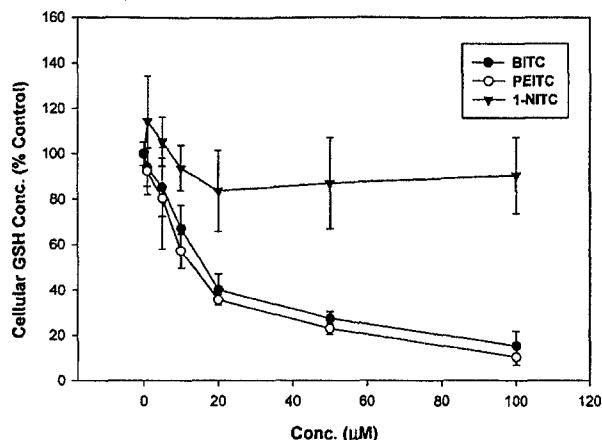


Figure 6. The effects of BITC, PEITC, and 1-NITC on the cellular GSH concentrations in PANC-1 cells following a 2-h drug treatment. The data are expressed as mean \pm SD, $n = 6$.

after a 24-h incubation [PEITC: $110 \pm 3.0\%$ ($p < 0.001$); 1-NITC: $116 \pm 3.0\%$ ($p < 0.001$)]. BITC did not have any effect at either time point. The data indicated that the effects of ITCs on cellular GST activity were small in PANC-1 cells.

DISCUSSION

To our knowledge, very little is known concerning the reversal of MDR by organic ITCs. Our laboratory first reported that two dietary ITCs (BITC and PEITC) and one synthetic ITC (1-NITC) could increase the accumulation of DNM and vinblastine in MCF-7/ADR and PANC-1 cells.⁷ The present article further characterizes

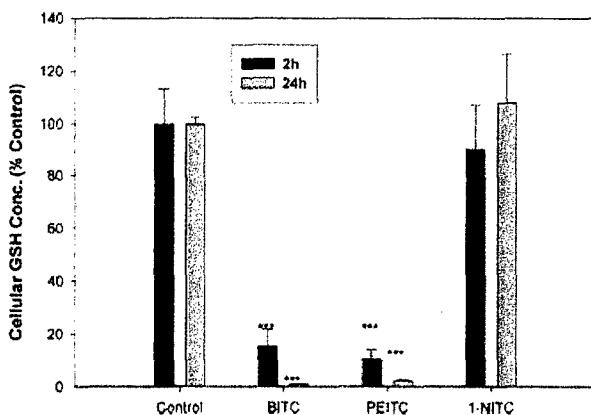


Figure 5. The effects of BITC, PEITC, and 1-NITC (100 μ M) on the cellular GSH concentration in PANC-1 cells following 2-h and 24-h drug treatments. The data are expressed as mean \pm SD, $n = 6$, *** $p < 0.001$.

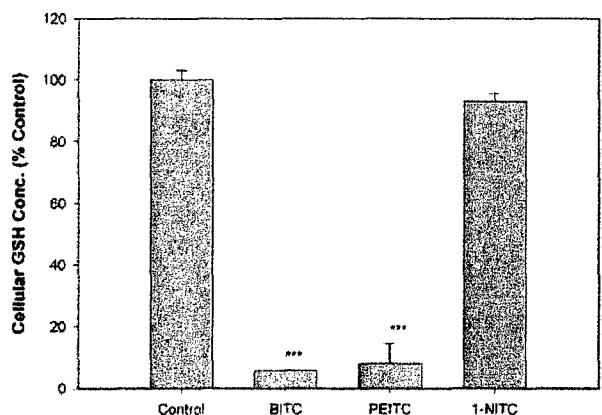


Figure 7. The effects of BITC, PEITC, and 1-NITC on the cellular GSH concentrations in Caco-2 cells following a 2-h drug treatment. The data are expressed as mean \pm SD, $n = 6$, *** $p < 0.001$.

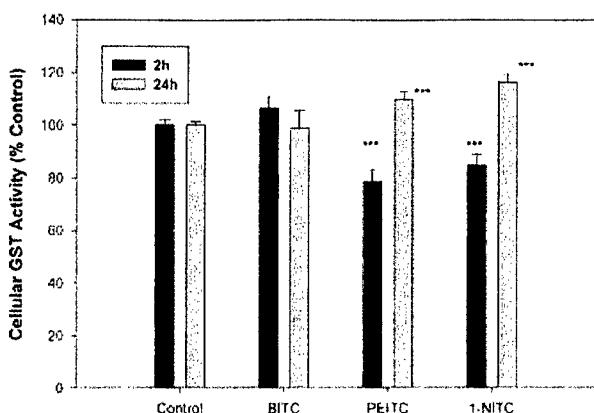


Figure 8. The effects of BITC, PEITC, and 1-NITC (100 μ M) on the cellular GST activity in PANC-1 cells following 2-h and 24-h drug treatments. The data are expressed as mean \pm SD, $n = 6$, *** $p < 0.001$.

this inhibition by examining other cell lines and evaluating potential mechanisms involved in these drug-transport interactions.

The isothiocyanate group ($-N=C=S$) of ITCs is highly reactive and hydrolyzes into isocyanate ($-N=C=O$) and amine ($-NH_2$) groups in aqueous matrix. It has been reported that BITC, PEITC, and 1-NITC undergo a spontaneous hydrolysis nonenzymatically, forming the corresponding amines EA, PA, and 1-NA via the intermediate isocyanates BIC, PEIC, and 1-NIC, respectively.¹⁶⁻¹⁸ The stabilities of the three ITCs in buffer were determined to verify whether transport interactions were due to the parent drugs or due to the isocyanates or amines. In the sodium buffer used in the current investigation, we found the BITC, PEITC, and 1-NITC exhibited good stability over 2 and 24 h periods. The apparent loss of parent drugs was mostly due to nonspecific binding to Falcon polystyrene 35 \times 10-mm dishes. Moreover, only parent ITCs, rather than ICs and amines, had effects on the accumulation of DNM in MCF-7/ADR, Caco-2, and PANC-1 cells.

The results of our studies indicate that PEITC and/or its radiolabeled metabolites are not substrates for P-gp. Studies performed in MDA435/LCC6MDR1 cells, which overexpress P-gp but do not have any detectable expression of MRP1, indicated that intracellular ^{14}C levels were similar in wild-type cells, which have little expression of P-gp, and in the transfected MDR1 cells. Lower intracellular concentrations of ^{14}C would be expected in P-gp overexpressing cells, if PEITC and/or its metabolites represent substrates. Addition-

ally, the P-gp inhibitor verapamil did not alter the accumulation of the ^{14}C radiolabel in these cells, again indicating that PEITC or its metabolites are not substrates for P-gp. The mechanism of the inhibition of P-gp-mediated efflux of DNM is unknown. Our results contrast with those of Zhang and Calloway,¹⁹ who suggested that the ITC sulforaphane is actively effluxed from cells by P-gp in the form of a GSH conjugate. ITCs are known to covalently bind to proteins through preferentially reacting with sulfhydryl groups of amino acid residues; whether this occurs *in vivo*, at relevant concentrations, is not known. ITCs can react with sulfhydryl and amino groups and less commonly hydroxyls.^{20,21} Fluorescein ITC has been shown to be a potent inhibitor of Na^+-K^+ -ATPase and gastric ATPase, but only a weak inhibitor of P-gp ATPase.^{20,22} Therefore, effects of ITCs may involve interactions at the ATP or at substrate binding sites, resulting in inhibition of P-gp-drug transport; however, proof of these interactions requires further experimental validation.

The results of this investigation identified two potential mechanisms involved in the inhibition of MRP1-mediated efflux. First, in PANC-1 cells, that overexpress MRP1 and exhibit no detectable expression of P-gp, the MRP inhibitor MK571 could increase the accumulation of the radiolabel. PEITC and BITC form conjugates with GSH catalyzed by GSTs, followed by hydrolysis of the resulting conjugates to the cysteine derivative and finally N-acetylation to form N-acetylcysteine (mercapturic acid) conjugates.²³ The presence of the glutathione conjugate appears to be important for the inhibition of MRP1 by the isothiocyanate, sulforaphane.²⁴ It has been reported that sulforaphane accumulates in mammalian cells by up to several hundred-fold over the extracellular concentration, primarily by conjugation with intracellular GSH, with intracellular concentrations in the mM range. MRP1 can either actively transport molecules conjugated to GSH such as leukotriene C₄, dinitrophenol, aflatoxin B1, chlorambucil, and melphalan²⁵ or actively cotransport GSH and the unmodified molecules out of the cell such as vincristine, etoposide, doxorubicin, and DNM.²⁶ It is likely that PEITC-GSH and BITC-GSH may compete for MRP1-mediated efflux. Second, incubation of PANC-1 or Caco-2 cells with PEITC or BITC for 2 or 24 h resulted in pronounced depletion of intracellular GSH. Buthionine sulfoximine treatment can reverse the MRP1-mediated efflux of some natural product drugs by depleting

cellular GSH concentrations. Recently, it was found that DNM was cotransported by MRP1 with GSH in a stoichiometric ratio of 1:1.²⁷ Based on the results of reported studies with sulforaphane,^{19,24} it is likely that PEITC and BITC will deplete intracellular GSH concentrations due to the formation of their glutathione conjugates. Whether there will be an effect on glutathione efflux, such as that produced by verapamil or by flavonoids,²⁸ is unknown.

Recently, a model describing the fate of ITCs and their conjugates was proposed by Thornalley.²⁹ It was proposed that BITC and PEITC enter cells by diffusion, but are almost entirely conjugated with GSH in cells (nonenzymatically and/or enzymatically).^{24,30} The intracellular ITC-SG may be further metabolized (such as by binding cellular proteins and the formation of other metabolites via mercapturic acid pathway), hydrolyzed to liberate ITCs, and GSH due to the reversible conjugation³¹ or predominantly (if not entirely) rapidly exported by an anion transporter such as MRP1 or MRP2.¹⁹ However, the half-lives of BITC-SG and PEITC-SG are short [67 and 58 min in phosphate buffer (pH 7.4), respectively³² or 44 min in PBS for PEITC-SG³³]. Therefore, ITC-SGs will rapidly degrade to ITCs and GSH extracellularly, allowing the ITC to reenter the cells by passive diffusion. As a result, the intracellular accumulation of ITCs will be maintained by a continuous uptake of ITCs to further bind cellular GSH, resulting in a depletion of GSH, based on this cycling model.²⁹

On the other hand, 1-NITC can inhibit the MRP1-mediated efflux of DNM, but did not significantly change the concentration of GSH in PANC-1 or Caco-2 cells. It has been suggested that 1-NITC enters cells by passive diffusion where it forms a reversible S-conjugate with GSH.³⁴ However, the conjugate of 1-NITC-SG is highly unstable (the half-life is less than 1 min).³⁴ Therefore, the GSH conjugate of 1-NITC is much less stable than BITC-SG and PEITC-SG,^{17,32} and, unlike BITC and PEITC, GSH and mercapturic acid conjugates have never been reported to be present in plasma or urine samples following 1-NITC administration.^{18,35} Alternatively, 1-NITC is enzymatically hydrolyzed to form 1-NA via 1-NIC as the intermediate,³⁵ predominantly mediated by CYP1A1,¹⁶ and the major metabolite found in the urine in rats is 1-NA.¹³ It has been proposed that 1-NITC-SG can be formed in the liver, and would be actively exported by MRP2 from hepatic parenchymal cells to bile,^{36,37} and that the dissociation of 1-NITC-SG in the bile could result in

an elevation of GSH concentration and exposure of bile duct epithelial cells and periportal parenchymal cells to large and possibly toxic concentrations of 1-NITC. This may represent one mechanism for the reported hepatotoxicity of 1-NITC.³⁷ Recently, Dietrich and coworkers found that 1-NITC-SG was actively transported by MRP2 in the canine kidney cell line MDCK II transfected by the human *MRP2* gene.³⁸ Moreover, the authors of that study found the depletion of cellular GSH concentration was due to a cycling of 1-NITC/1-NITC-SG, similar to the proposed mechanism for BITC and PEITC.²⁴ However, in this study, 1-NITC did not change cellular concentrations of GSH in either PANC-1 or Caco-2 cells. It is possible that the lack of effect of 1-NITC in Caco-2 cells may be due to its rapid metabolism in this cell line, because 1-NITC also did not alter the accumulation of DNM in Caco-2 cells. However, 1-NITC was an effective inhibitor of the MRP1-mediated efflux of DNM in PANC-1 cells: its activity appears unrelated to GSH intracellular concentrations, but may reflect inhibition of transport by its GSH conjugate or by sulfhydryl binding interactions. These results strongly indicate that the mechanism involved in MRP1 inhibition by 1-NITC may differ, at least in part, from that of BITC and PEITC.

Studies have reported that BITC and PEITC can induce GST.^{5,39} In this study, GST activity was decreased (15–21%) by the treatment of PEITC and 1-NITC for 2 h, and increased approximately 10–16% after 24-h treatment of the same drugs, compared to the control. However, BITC did not change GST activity at either time point. These differences may reflect cell-dependent differences reported by Jiang et al.,³⁹ as well as concentration-dependent or time-dependent differences. Whether the small changes in GST activity observed in this investigation contribute to the effect of these compounds on MRP1-mediated efflux is unknown.

CONCLUSION

In conclusion, we found that BITC, PEITC, and 1-NITC increase the accumulation of DNM in several P-gp- and MRP1-overexpressing cell lines. The activities were due to the parent isothiocyanates; the corresponding isocyanate and amine metabolites had no activity. PEITC and/or its conjugated metabolites were not substrates for P-glycoprotein, but the glutathione conjugate of PEITC likely represents a substrate of MRP1.

Effects of the ITCs on GST activity in PANC-1 cells were small, but depletion of cellular GSH concentrations occurred after treatment with PEITC and BITC, but not with 1-NITC. This suggested that the mechanism of BITC and PEITC effects on MRP1 may differ from that of 1-NITC. We propose that the reversal activities of BITC and PEITC for MRP1 are due, at least in part, to the depletion of cellular GSH concentration as a cosubstrate in DNM efflux and to the competitive binding of the GSH conjugates of PEITC and BITC to a MRP1 substrate binding site. The lack of effect of 1-NITC on DNM accumulation in Caco-2 cells was most likely due to the extensive metabolism of 1-NITC in this cell line.

ACKNOWLEDGMENTS

Financial support for this study was provided by the grants from the New York State Health Research Science Board (EMPIRE grant), the Susan G. Komen Breast Cancer Foundation, the Kapoor Charitable Foundation (University at Buffalo) and the U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376. We acknowledge Qi Wang for his synthesis and characterization of ¹⁴C-PEITC.

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Appendix 6

Efflux Transporters in Drug Excretion

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Introduction

Therapeutic agents or other xenobiotic compounds will exert their pharmacological or toxicological activities only when sufficient concentrations of these compounds are present at the site of action, where they can bind to the targeted receptors or enzymes. Therefore, the ability of drug molecules to cross biological membranes represents an important determinant of their absorption, distribution, elimination and ultimately their therapeutic or toxic effects. It is clear that the complex biological membrane system is not just pure lipid bilayers, but lipid bilayers embedded with numerous proteins, including transporters. Thus, for a large number of drug molecules, their ability to pass through biological membranes is not solely determined by their physiochemical parameters such as lipophilicity, but also governed by the transporter activities. Among these transporters, a group of so-called efflux transporters (Table 1), including P-glycoprotein, multidrug resistance-associated proteins (MRPs) and breast cancer resistant protein (BCRP) are of particular interest in that they actively remove a wide range of structurally and functionally distinct molecules out of the cells against a concentration gradient. Their transport activities towards a number of clinically important anticancer agents, such as doxorubicin, paclitaxel and vinblastine prevent the intracellular accumulation of these cytotoxic agents and lead to inefficient cell killing, a phenomenon known as multidrug resistance (MDR), which remains the primary obstacle to the successful cancer chemotherapy (1-4). In addition, studies characterizing the molecular and functional properties and physiological functions of these transporters, have revealed that these efflux transporters, apart from mediating MDR, play an essential role in governing the absorption, and the intestinal, hepatobiliary and renal excretion of a variety of endogenous and exogenous

compounds (5-9). The localization of these efflux transporters in the luminal side of the blood-brain barrier, blood-testis barrier and placenta suggests their central role in regulating the entry of potentially harmful compounds into these pharmacological sanctuaries. It is widely accepted that at least some of these transporters constitute an essential component for the barrier functions between the blood and various tissues and determine the passage of drug molecules or other compounds into these tissues (5-9). Furthermore, considering the impact of these transporters on drug disposition, their wide substrate spectrum and their potential saturability, adverse drug interactions due to competitive inhibition or induction of these efflux transporters by coadministered drugs, ingested food or environmental compounds could be expected, and this has been proven in a number of *in vivo* animal or clinical studies (10-14). On the other hand, these transport interactions, may also result in beneficial interactions and improve the therapeutic efficacy of a particular drug of interest. For example, the poor bioavailability of some anticancer agents could be improved by inhibiting intestinal P-glycoprotein or other efflux transporters (15-17). Lastly, it has been shown that the expression of these efflux transporters varies substantially between individuals, and this variability could be due to the age and gender difference, genetic polymorphism or prior exposure to drugs, food and environmental compounds (13, 18-22). The impact of this variability in the expression of these transporters on drug pharmacokinetics remains the topic of extensive investigation and the results obtained from these studies will have significant impact on future therapy. To appreciate the importance of the efflux transporters in drug therapy, an understanding of the molecular and functional characteristics of these transporters and their tissue distribution as well as an appreciation of their impact on drug disposition is essential. This is the focus of the present overview.

1. P-glycoprotein

P-glycoprotein is a membrane efflux transporter protein discovered by Juliano and Ling in 1976 from the plasma membrane of Chinese hamster ovary cells selected for resistance to colchicine (23). These cells also displayed pleiotropic cross-resistance to a wide range of amphiphilic drugs with distinct

structure and function, a phenomenon nowadays known as multidrug resistance (MDR). The consistent observation of this membrane protein in several MDR cell lines selected with different drugs (23-26) and the positive correlation found between the level of P-glycoprotein expression and drug resistance in a variety of MDR cell lines (27, 28) strongly suggested that P-glycoprotein may play a key role in mediating MDR. This was subsequently confirmed by gene transfer studies (29, 30), in which transfection of P-glycoprotein cDNA was shown sufficient to confer the MDR phenotype upon otherwise drug sensitive cells. The mechanism by which P-glycoprotein mediates MDR is believed to be that P-glycoprotein functions as an ATP-dependent efflux pump, actively extruding a wide range of cytotoxic agents, such as anthracyclines, vinca alkaloids, epipodophyllotoxins and taxol, from inside the cell to the extracellular space, resulting in inadequate intracellular accumulation of these agents for efficient cell killing (1, 31-34). It is well established that P-glycoprotein overexpression is one of the major mechanisms responsible for the development of MDR (2, 35). The clinical relevance of this MDR mechanism was substantiated by the findings that P-glycoprotein was often detected in numerous resistant human tumors and the expression of this protein represents a poor prognosis factor (36-44).

The genes encoding P-glycoprotein have been cloned and belong to a small family of closely related genes designated as *mdr*. The family consists of two members (MDR1 and MDR3) in humans, and three members (*mdr1a*, *mdr1b* and *mdr2*) in rodents (45-48). Despite the high homology shared between different members of the family, only human MDR1 and its mouse homologue *mdr1a* and *mdr1b* protein can confer multidrug resistance and drug transport capabilities, while human MDR3 and its mouse homologue *mdr2* protein apparently can not (29, 30, 47, 49-54). The latter was shown to be more concentrated in the liver canalicular membranes and functions as a phosphatidylcholine translocase or flippase (55-58). Human P-glycoprotein has 1280 amino acids and the polypeptide component of the protein has a molecular weight of 120 to 140 KD (45). The apparent molecular weight of P-glycoprotein, however, could vary between 130-190 KD, depending on the level of glycosylation. The molecular structure of the protein was predicted to consist of two homologous halves, each consisting of six transmembrane

domains, and a hydrophilic nucleotide binding domain with Walker A, Walker B and ABC signature sequences, characteristic of ABC proteins (Fig. 1). The nucleotide binding sites are located intracellularly and exhibit ATPase activity, which hydrolyzes ATP and provides the energy for the pumping function of the protein (59, 60).

One of the distinctive features of P-glycoprotein from conventional drug transporters is its broad spectrum of substrate specificity (Table 2). These substrates include anticancer agents (e.g., anthracyclines, vinca alkaloids, epipodophylotoxins and taxol) (2), cardiac drugs (e.g., digoxin, quinidine) (61, 62), HIV protease inhibitors (e.g., saquinavir, indinavir, ritonavir) (63), immunosuppressants (e.g., cyclosporine) (64), antibiotics (e.g., actinomycin D) (65) steroids (e.g. cortisol, aldosterone, dexamethasone) (66, 67) and cytokines (e.g., IL2, IL-4, IFN- γ) (68). The list of P-glycoprotein substrates could be expanded to include many more compounds. The only common characteristics of these substrates are that most of these compounds are hydrophobic, positively charged or neutral compounds with planar structure (2, 69); however, negatively charged compounds, such as methotrexate and phenytoin, can also serve as substrates under certain circumstances (70-72). How P-glycoprotein recognizes such a wide range of structurally unrelated chemical entities still remains an enigma, but could be partly owing to the multiple drug binding sites present in the transmembrane domains of the protein (73-76). The proposed mechanism by which P-glycoprotein performs its transport function is the so-called "hydrophobic vacuum cleaner" model or the "flippase" model (2, 77, 78). In the "hydrophobic vacuum cleaner" model, P-glycoprotein binds directly to its substrates within the plasma membrane and pump them out of the cells (2). In the "flippase" model, the binding of substrates takes place in the inner leaflet of the plasma membrane bilayer and the substrates are flipped by P-glycoprotein to the outer leaflet, from which they diffuse into the extracellular space (77, 78). In either case, the substrates are removed directly from the cell membrane by P-glycoprotein before their entry into the cytoplasmic solution. The high local concentrations of the hydrophobic compounds in the lipid membrane may facilitate the transport by P-glycoprotein even in the absence of high affinity binding, and this may also help to explain such a diverse substrate spectrum (79).

A wide range of P-glycoprotein inhibitors, that are as chemically diverse as the substrates, has also been identified. These inhibitors include calcium channel blockers (e.g., verapamil, diltiazem) (80), calmodulin antagonists (e.g., trifluoperazine, fluphenazine) (81, 82), steroid compounds (e.g., progesterone, tamoxifen) (83, 84), immunosuppressive agents (e.g., cyclosporin A, FK506) (85, 86), antibiotics (e.g., cefoperazone, erythromycin) (87, 88) and nonionic detergents (e.g., Triton-X100, Nonidet P-40) (89). Interestingly, a number of pharmaceutical excipients such as cremophor EL, Tween 80, and polyethylene glycols were also shown to inhibit P-glycoprotein (90, 91). More recently, the list of these inhibitors has been extended to include many dietary compounds in a variety of natural products, such as flavonoids (92-95), curcumin (96) and piperine (97). Many of these inhibitors have undergone clinical testing for their ability to restore tumor responsiveness to chemotherapeutic agents by blocking P-glycoprotein; however, the toxicities associated with the high concentrations of these inhibitors required for a significant P-glycoprotein inhibition have prevented their clinical use. The newly-developed second and third generations of P-glycoprotein inhibitors such as PSC833 (98), GF120918 (99), LY335979 (100) and XR9576 (101) have very high potency and low toxicity, and clinical trials using these agents as chemosensitizers have produced some promising results (102-105).

The expression of P-glycoprotein is not limited to MDR tumor cells. High levels of expression have been also detected in a number of normal tissues, such as the liver, kidney, gastrointestinal tract, the blood-brain and blood-testis barriers, as well as the adrenal glands (106-109). At the subcellular level, P-glycoprotein has been shown to be predominantly located on the apical surface of the epithelial (or endothelial) cells with a specific barrier function, such as the endothelial cells of the blood capillaries in the brain, the canalicular membranes of the hepatocytes, the brush border membranes of renal proximal tubules, and the luminal membrane of the enterocytes in the colon and jejunum (106, 108, 109). The polarized expression of this protein in the excretory organs (liver, kidney and intestine) and blood-tissue barriers, together with its ability to transport a wide diversity of chemicals, indicates that the protein may play an important role in protecting the body or certain tissues (such as brain and testis)

from the insult of ingested toxins and toxic metabolites, by actively excreting these toxic agents into bile, urine and intestine, or by restricting their entry into the brain and other pharmacological sanctuaries. P-glycoprotein was also found in placental trophoblasts from the first trimester of pregnancy to full term, indicating it may be also involved in the protection of the developing fetus (2).

The role of P-glycoprotein in manipulating excretion and distribution of xenobiotics was initially supported by a number of *in vivo* animal or clinical studies using a combination of P-glycoprotein substrate drugs and inhibitors, in which a reduced elimination and increased tissue accumulation of the substrate drugs by the co-administered inhibitors were often observed (110-112). However, due to the possible interactions between these inhibitors and other drug transporters or drug metabolizing enzymes and since the inhibitors used in these early studies were relatively non-specific, other interpretations could not be excluded. The most convincing evidence is from a series of elegant studies conducted by Schinkel et. al. (62, 113, 114), using knockout mice. Both *mdr1a* (-/-) and *mdr1a/1b* (-/-) knockout mice have been created by disruption of *mdr1a*, or both *mdr1a* and *mdr1b* genes. These knockout mice appeared to be viable, healthy and fertile with normal histological, hematological, and immunological parameters, indicating that mdr1-type P-glycoprotein may not be essential for basic physiology (113, 114). However, the mice lacking mdr1 type P-glycoprotein did show hypersensitivity to xenobiotic toxins. For example, the *mdr1a* (-/-) mice were 50-100-fold more sensitive to ivermectin, an acaricide and antihelmintic drug, compared to the wild type mice, and this increased toxicity could be explained by the 90-fold increase in the brain accumulation of ivermectin in the knockout mice, since the toxicity of ivermectin results from its interaction with a neurotransmitter system in the central nervous system (CNS) (113). Another interesting example is related to the antidiarrheal drug loperamide, which is a P-glycoprotein substrate. Although loperamide is a typical opioid drug, in humans and animals this drug only demonstrates peripheral opiate-like effects on the gastrointestinal tract with little effect in the CNS due to its inability to pass through the blood brain barrier. After oral administration of loperamide, the *mdr1a* (-/-) mice demonstrated markedly increased CNS opiate-like effects compared with the wild type mice, consistent with a dramatic increase in the brain accumulation of

this drug in the knockout mice (13-fold, $p < 0.001$) (115). Interestingly, CNS effects of loperamide in humans were also observed when it was co-administered with quinidine, a competitive inhibitor of P-glycoprotein (10). An increased brain accumulation of many other P-glycoprotein substrate drugs such as vinblastine, cyclosporine, digoxin have also been observed in the *mdr1a* (-/-) or *mdr1a/1b* (-/-) mice (62, 113-115). Taken together, these data clearly indicate that mdr1-type P-glycoprotein plays a very important role in regulating the entry of xenobiotics or endogenous compounds into the brain. In addition to the marked alterations in the brain accumulation of these P-glycoprotein substrates in the knockout mice, the blood concentrations and the accumulation of these substrates in other tissues such as the liver, heart and intestine were also shown to be significantly elevated, albeit to a lesser extent, indicating a diminished elimination of these compounds in the knockout mice (62, 113-117). The high level of P-glycoprotein found in the excretory organs in the body and the diminished elimination of P-glycoprotein substrates observed in P-glycoprotein-deficient mice point to an important role of the protein in the elimination of xenobiotics by these excretory routes.

Xenobiotics can be eliminated from the body by fecal excretion if they are poorly absorbed after oral administration or following secretion into the intestinal lumen. The polarized expression of P-glycoprotein on the apical membrane of the enterocytes lining the intestinal wall (106) suggests this efflux transporter is involved in the active secretion of P-glycoprotein substrates into the intestinal lumen and thus facilitates their fecal excretion. In addition, the P-glycoprotein-mediated active efflux of its substrates from the intestinal epithelial cells back to the lumen will also limit the absorption / bioavailability of orally dosed drugs or other compounds that are P-glycoprotein substrates. Significant P-glycoprotein-mediated effects on intestinal secretion and absorption / bioavailability have been observed in a number of studies. In mice, *mdr1a* P-glycoprotein is the major isoform expressed in the intestine and brain (48, 113). The plasma AUC of paclitaxel, a known P-glycoprotein substrate, has been shown to be 2- and 6-fold higher in *mdr1a* (-/-) knockout mice than in the wild type mice after i.v. and oral administration, respectively. The cumulative intestinal secretion of paclitaxel (0-96 hour) was dramatically decreased from 40% in the wild type animal to < 3% in the knockouts after i.v.

dosing, and the bioavailability of paclitaxel increased from 11% in the wild type mice to 35% in the knockouts after oral dosing (10 mg/kg) (118). Similar results have also been obtained for a number of other P-glycoprotein substrates, such as digoxin, grepafloxacin, vinblastine and HIV protease inhibitors (119-123). For example, the direct intestinal secretion of ³H-digoxin was only 2% of the dose in *mdrla* (-/-) mice, in contrast to 16% in the wild type animals (119). Collectively, the results obtained from these knockout animal studies provide convincing evidence for the important contribution of P-glycoprotein to intestinal secretion and absorption of substrate compounds. The clinical relevance of these observations in the animal studies has been demonstrated in several human studies. For example, the intestinal secretion of talinolol, a β_1 -adrenergic receptor blocker, was shown to be against a concentration gradient (5.5 (lumen): 1 (blood)), after its i.v. administration, indicating the involvement of an active process. In addition, the secretion rate of talinolol in the presence of a simultaneous intraluminal perfusion of R-verapamil, a known P-glycoprotein inhibitor, dropped to 29-59% of the values obtained in the absence of R-verapamil (124). Similar results have also been obtained for digoxin (125). Furthermore, the intestinal secretion of talinolol was also increased significantly in human subjects treated with rifampin, and the increased secretion can be attributed to the 4.2-fold increase in the intestinal P-glycoprotein expression induced by treatment with rifampin (13). The oral bioavailability of P-glycoprotein substrates in humans was also shown to be, at least partly, limited by intestinal P-glycoprotein (20, 126-131), and co-administration of P-glycoprotein inhibitors or competitive substrates could increase the bioavailability of these substrates (11, 12).

Biliary excretion represents another important route for the elimination of drugs and other xenobiotics. Following the uptake of xenobiotics into the hepatocytes, compounds may undergo metabolic modification, or the parent compound, as well as the formed metabolites may be excreted into bile through the canalicular membrane or effluxed back across the sinusoidal membrane into blood. The relatively small surface area of the canalicular membrane (10-15% of the hepatocyte surface area) in contrast to the sinusoidal membrane (at least 70%) and small intracanalicular fluid volume suggests that carrier mediated transport may significantly contribute to the biliary excretion of both

endogenous and exogenous compounds (7, 132). Indeed, many active transporters have been identified in the canalicular membrane to mediate this process (132-134), including P-glycoprotein and MRP2 (106, 132). The contribution of P-glycoprotein to biliary secretion has been demonstrated by several investigations. For example, the biliary excretion of unchanged doxorubicin decreased from 13.3% of the dose in wild type mice to only 2.4% in *mdr1a* (-/-) knockout mice after a 5 mg/kg i.v. dose. (121). Similar results have also been obtained for a number of amphiphilic model substrates, which exhibited markedly reduced biliary excretion in both *mdr1a* (-/-) and *mdr1a/1b* (-/-) knockout mice compared to the normal mice (117, 123). Studies using P-glycoprotein inhibitors also provided results consistent with the important contribution of P-glycoprotein to biliary excretion. In an isolated perfused rat liver study, erythromycin significantly decreased the biliary excretion of fexofenadine, which is a P-glycoprotein substrate (135). Cyclosporin A and its analogue PSC833 have been reported to decrease the biliary excretion of both colchicine and doxorubicin (136, 137) in vivo. Similar results have also been observed for doxorubicin and grepafloxacin when the competitive substrates erythromycin (for both doxorubicin and grepafloxacin) and cyclosporin (for grepafloxacin) were administered simultaneously (138, 139). In addition, the biliary excretion of P-glycoprotein substrates was shown to depend on the expression level of this protein, and a significant increase in the biliary excretion of vinblastine was observed in rats with increased levels of P-glycoprotein, which was induced by 2-acetylaminofluorene and phenothiazine, respectively, in two independent studies (140, 141). These data suggest that P-glycoprotein plays an important role in biliary excretion. However, other studies have failed to find significant effects on P-glycoprotein-mediated biliary excretion in knockout mice. For example, while the intestinal secretion and bioavailability of paclitaxel were markedly altered in *mdr1a* (-/-) knockout mice, the biliary excretion of this model substrate in the knockout mice was not significantly different from that in the wild type animals (118). Even in the *mdr1a/1b* (-/-) double knockouts, the biliary excretion of both digoxin and vinblastine was not substantially changed (114). One possible explanation of these conflicting results is the presence of alternative transport processes responsible for the secretion of these substrates into bile. P-glycoprotein may act in concert with other transporters in excreting certain substrates into bile,

and the loss of P-glycoprotein function could be compensated for by other transport processes under certain circumstances. Indeed, it has been shown that *mdr1b* expression in the liver and kidney was consistently increased in *mdr1a* (-/-) knockout mice compared to the wild type animals, indicating that the loss of *mdr1a* function could be compensated for by *mdr1b* protein for their common substrates (113). Other canalicular membrane transporters may also exhibit overlapping substrate specificity for certain P-glycoprotein substrates.

Renal clearance represents an important route for the elimination of a large number of xenobiotic compounds. This dynamic process includes glomerular filtration, renal tubular secretion and tubular reabsorption. Renal secretion usually takes place against a concentration gradient and thus is mainly an active process involving a variety of transporter mechanisms (142). In addition to the two major carrier systems responsible for the renal handling of organic cations and organic anions, several ATP-dependent transporters, including P-glycoprotein and multidrug resistance associated proteins have been detected in the kidney (142). The transport function and the localization of P-glycoprotein on the apical membrane of the proximal tubule cells (106) suggest the involvement of this protein in the renal secretion of its substrates into urine. The observation that a classic P-glycoprotein inhibitor, cyclosporin, decreased colchicine renal clearance after i.v. administration from 6.23 ± 0.46 to 3.58 ± 0.31 ml/(min•kg) (mean \pm SD, $p < 0.05$) without affecting glomerular filtration and the secretion of the organic cation ranitidine or the organic anion p-aminohippurate, provided the first *in vivo* demonstration for this functional role of P-glycoprotein (143). Subsequently, a significant reduction of the renal secretion of digoxin (in rats), vinblastine and vincristine (in dogs) by cyclosporin A was also observed by using the isolated perfused rat kidney or the single pass multiple indicator dilution method (144, 145). In humans, the renal clearance of digoxin was decreased by the concomitant use of itraconazole by 20% ($p < 0.01$). Since digoxin is mainly excreted unchanged into urine, this reduction is most likely mediated by the inhibition of P-glycoprotein (146). Similarly, the renal clearance of quinidine was also decreased by 50% ($p < 0.001$) by itraconazole in a double-blind, randomized, two-phase crossover study, and inhibition of P-glycoprotein is thought to be the most likely underlying mechanism (147). Taken together, these studies

demonstrated that P-glycoprotein significantly contributes to the renal excretion of its substrates.

2. Multidrug Resistance-associated Protein (MRP)

The family of human multidrug resistance associated proteins (MRP) is another group of ABC transporters, so far consisting of nine members. Among these members, MRP1, MRP2 and MRP3 have been characterized in some detail in terms of their capability of conferring multidrug resistance and their possible physiological functions (148) and so will be the focus of this discussion. The founding member of this family, MRP1, was cloned in 1992 from the resistant human small cell lung cancer cell line (149), which does not overexpress P-glycoprotein (150-153). Subsequent transfection studies demonstrated that overexpression of this 190 KD membrane protein can confer multidrug resistance against a number of natural product anticancer agents such as the anthracyclines, vinca alkaloids and epipodophyllotoxins, by causing the active efflux of these cytotoxic agents from cells and thus lowering their intracellular concentrations (154-157). Later, MRP2 (cMOAT) and other members were also identified and characterized to varying extents (158-167). Among these MRPs, MRP3 is the most closely related member to MRP1 with 58% amino acid identity, followed by MRP2 (49%) (168). These three MRPs have similar topology, containing a typical ABC core structure of two segments with each consisting of 6 transmembrane domains and an ATP binding domain, similar to P-glycoprotein, and an extra N-terminal segment of five transmembrane domains linked to the core structure through an intracellular loop (148) (Fig. 1). Similar to MRP1, both MRP2 and MRP3 have also been shown to be able to confer MDR to several anticancer drugs (169-172). The clinical relevance for MRP1-mediated MDR has been a topic of extensive investigation and there is some evidence suggesting that overexpression of MRP1 might represent a poor prognostic factor (173-181). The clinical relevance of MRP2- and MRP3-mediated MDR is currently unknown.

In contrast to P-glycoprotein, which mainly transports large, hydrophobic cationic compounds, MRP1 mainly transports amphiphilic anions,

preferentially lipophilic compounds conjugated with glutathione (e.g., leukotriene C4, DNP-SG), glucuronate (e.g., bilirubin, 17 β -estradiol), or sulfate (5) (Table 2). Some unconjugated amphiphilic anions such as methotrexate and Fluo-3, a penta-anionic fluorescent dye, can also serve as substrates and they are transported in unchanged form (182, 183). In addition to the anionic compounds, MRP1 can also accept amphiphilic cations or neutral compounds, such as anthracyclines, etoposide and vinca alkaloids, as its substrates. But paclitaxel, which is a good P-glycoprotein substrate, appears not to be transported (154-157). These cationic or neutral substrates are thought to be transported intact but need reduced glutathione (GSH) as a cotransporting factor (184-187). As such, depletion of intracellular GSH by buthionine sulphoximine (BSO), an inhibitor of glutathione (GSH) synthesis, can increase the intracellular accumulation of these substrates in MRP1 overexpressing cells (188, 189). Both MRP2 and MRP3 share a similar substrate spectrum with MRP1. They can also transport conjugates of lipophilic substances with glutathione, glucuronate and sulfate such as glutathione S-conjugate leukotriene C4, glucuronosyl bilirubin and anticancer agents methotrexate, vincristine and etoposide (5). In transporting cationic substrates, MRP2 and MRP3 seem to function by the same mechanism as MRP1 and need GSH as a cosubstrate (187). However, the substrate specificity of these three MRP isoforms is not identical, and for their common substrates, the transporting efficiency by these isoforms varies substantially (5); there are substrates that can be recognized by one isoform but not the others. For example, cisplatin has been shown to be a substrate for MRP2 but not for MRP1 (154, 190, 191); the conjugated monoanionic bile acids glycocholate and taurocholate are substrates for MRP3 but not for MRP1 and MRP2 (192-194). In contrast to P-glycoprotein, for which many inhibitors have been identified, there are only a few compounds known to inhibit MRP to a significant degree. The well-known potent P-glycoprotein inhibitors such as GF120918 and LY335979 have little effect on MRP while verapamil, cyclosporin A and PSC833 have been shown to be, at best, moderate MRP inhibitors (5, 195, 196). The best-known MRP inhibitor so far appears to be MK571, which is a leukotriene D4 receptor antagonist. MK571 inhibits both MRP1 and MRP2, but a mild stimulatory effect on MRP3-mediated transport of 17 β -estradiol glucuronide has been reported (159, 197, 198).

To understand the physiological function of these energy-dependent MRP efflux transporters, their normal tissue distribution has been extensively investigated. While MRP1 appears to be distributed in a wide range of tissues throughout the body, MRP2 and MRP3 have been detected mainly in the gut, liver and kidney (6, 148). At the subcellular level, MRP1 is predominantly located in the cell plasma membrane, and in polarized epithelial cells such as hepatocytes, enterocytes and endothelial cells, its distribution is confined to the basolateral membranes (6, 148, 199). The active transport function of MRP1 towards a number of exogenous and endogenous toxic substrates and its ubiquitous tissue distribution indicate that MRP1 may represent a detoxifying mechanism, protecting some tissues or organs from exposure to toxic substances (168). Recent studies using *mrp1* (-/-) knockout mice have provided convincing evidence for this important function. It has been shown that mice with disrupted Mrp1 (*Mrp1* (-/-)) are viable, fertile and have no physiological or histological abnormalities, indicating that Mrp1 may be not essential for normal mouse physiology. However, these *mrp1* (-/-) mice did show a two-fold higher sensitivity to a cytotoxic agent, etoposide, with increased bone marrow toxicity (200, 201). A similar observation has also been made by Johnson et. al. (202), who demonstrated that a therapeutic dose of vincristine, which normally does not express bone marrow toxicity and gastrointestinal damage, caused extensive damage to these tissues in both *mrp1* (-/-) and *mdrla/1b* (-/-) knockout mice, indicating that Mrp1, mdrl-type P-glycoprotein and probably other related efflux transporters work in concert as a detoxifying mechanism to protect tissue from damage induced by toxic agents. In addition, the polarized localization of MRP1 in the basolateral membrane of the choroid plexus epithelium (203) suggests that it may significantly contribute to the blood-CSF (cerebrospinal fluid) barrier function, preventing the entry of amphiphilic anions or anticancer drug substrates into CSF. This has also been convincingly demonstrated in a knockout mice study conducted by Wijnholds et. al. (204), in which the investigators found that after an i.v. dose of etoposide, the CSF concentration was about 10-fold higher in *mdrla/mdrlb/mrp1* (-/-) triple knockout mice than in *mdrla/mdrlb* (-/-) double knockout mice, indicating the important contribution of Mrp1 to the blood-CSF barrier function in mice. Taken together, there is strong evidence indicating that MRP1 plays an important role in

protecting the tissue from the damage induced by both exogenous and endogenous toxic substances, and contributes significantly to maintaining the blood-CSF barrier function.

Unlike MRP1, the distribution of MRP2 and MRP3 are restricted to certain tissues such as liver, intestine and kidney (6, 148). Similar to P-glycoprotein, MRP2 is exclusively localized to the apical membrane of the polarized cells such as hepatocytes, intestinal epithelial cells and renal proximal tubule cells (5, 159, 205), suggesting it may also play a similar role in the secretion of xenobiotics and endobiotics by these excretory routes. The loss of MRP2 in humans is associated with Dubin-Johnson syndrome, a benign hereditary disorder characterized by mild conjugated hyperbilirubinemia and pigment disposition in the liver due to impairment in the MRP2-mediated transport function (206-209). Two naturally occurring mutants GY/TR and EHBR rats from the Wistar and Sprague-Dawley rat colonies, respectively, also lack Mrp2 expression and are considered animal models for the human Dubin-Johnson syndrome (158, 160, 210, 211). Many functional-characterization and substrate-identification studies for MRP2 have been performed by using these Mrp2 deficient rats. It has been shown that the AUC (0-6 hour) of ¹⁴C-temocapril was dramatically increased and the biliary clearance, as measured by total radioactivity, was markedly decreased (0.25 ml/min/kg vs. 5.00 ml/min/kg) in EHBR rats compared with the control Sprague Dawley rats after i.v. administration. Since the active metabolite temocaprilat accounted for > 95% of the total radioactivity, these data indicate that Mrp2 plays a central role in the biliary excretion of the metabolites of this drug (212). The biliary excretion of grepafloxacin was also markedly decreased for both parent compound (0.52 vs. 1.79 ml/min/kg) and glucuronide metabolites (0.09 vs. 15.53 ml/min/kg) in EHBR rats compared with the Sprague Dawley rats (213). Recently, Chen et al. (214) reported that the biliary excretion of methotrexate and probenecid was decreased 39- and 37-fold, respectively, in EHBR rats as compared to control rats. Similar results were also observed for several other drugs or metabolites such as cefodizime, acetaminophen glucuronide, acetaminophen glutathione conjugate and acetaminophen mercapturate, pravastatin, and indomethacin glucuronide (215-219). Interestingly, it was shown that the biliary excretion of CPT11, the active metabolite SN-38, and its glucuronide conjugate can be

substantially decreased by probenecid, an MRP2 inhibitor, with concomitant elevation of plasma concentrations of these compounds in normal rats, resulting in decreased GI toxicity (220). Collectively, these data strongly suggest the essential role of MRP2 in the biliary excretion of xenobiotics or their metabolites that are MRP2 substrates. The polarized localization of MRP2 in intestinal epithelial cells also suggests its potential contribution to intestinal secretion and to limiting the intestinal absorption of its substrates, leading to a decreased bioavailability. This hypothesis has been supported by the results of a number of studies. For example, after i.v. administration of CDNB (1-Chloro-2,4-dinitrobenzene), the intestinal secretion of DNP-SG (2,4-dinitrophenyl-*S*-glutathione) was negligible in EHBR rats, whereas a small amount of secretion was observed in Sprague Dawley rats, indicating the involvement of MRP2 in the active secretion of DNP-SG into intestinal lumen. This was also confirmed by Ussing chamber studies, in which the serosal-to-mucosal flux of DNP-SG was shown to be 1.5-fold higher than the mucosal-to-serosal flux in Sprague Dawley rats, and no difference in the flux in both directions was observed in EHBR rats (221). The decreased intestinal secretion of grepafloxacin in EHBR rats was observed in a study by Naruhashi et. al. (222), which was also confirmed by the 2-fold higher flux in the serosal-to-mucosal direction compared with that in the mucosal-to-serosal direction in the Sprague Dawley rats and no differences in the EHBR rats. By analogy with P-glycoprotein, the impact of MRP2-mediated efflux of its substrates from the enterocytes into the lumen can be illustrated by the 2-fold higher absorption of PhIP (p-2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine), a food-derived carcinogen, in Mrp2-deficient rats compared with the normal rats, and the increased bioavailability of PhIP in normal rats treated with BSO, which is a inhibitor for GSH synthesis (223, 224). All these studies provide convincing evidence for the important contribution of MRP2 to the intestinal secretion and absorption of drugs. Whether MRP2 is also present in the brain capillary endothelial cells may still need further investigation, but current evidence suggests it most likely is (225). Mouse Mrp2 was detected on the luminal surface of the brain capillary endothelium (226) and shown to actively transport sulforhodamine 101 and fluorescein methotrexate into the luminal compartment of isolated brain capillary (226). This transport process can be inhibited by leukotriene C₄, 1-chloro 2,4-dinitrobenzene (a precursor of DNP-SG) and vanadate (an ATPase

inhibitor), but not by P-glycoprotein inhibitors such as PSC833 and verapamil. Therefore, the evidence suggests that human MRP2 may also contribute to the blood-brain barrier function in a similar manner as P-glycoprotein does. MRP3 has a similar tissue distribution as MRP2, but is located on the basolateral surface of the polarized cells (171, 199, 227).

The expression of MRP3 in the basolateral membrane of intestinal epithelial cells, hepatocytes and renal proximal tubule cells suggests that it tends to remove the substrates from the cytosol into blood. The impact of this process on drug disposition still remains to be clarified, especially considering its limited expression in the excretory organs under normal physiological condition. Interestingly, it has been shown that MRP3 is significantly up-regulated in the liver of MRP2-deficient rats and in the patients with Dubin-Johnson syndrome or patients with primary biliary cirrhosis (227, 228), indicating MRP3 may serve as a compensatory mechanism to remove the conjugates out of the hepatocytes through sinusoidal membrane under the condition where the MRP2-mediated biliary excretion is impaired (5).

3. Breast Cancer Resistance Protein (BCRP)

BCRP is a new member of the ABC transporter superfamily initially cloned from a doxorubicin-resistant breast cancer cell line (MCF-7/AdrVp) selected with a combination of adriamycin and verapamil (229). Two other groups also independently identified this transporter from human placenta (230) and human colon carcinoma cells (S1-M1-80) (231), and named the protein ABCP (ABC transporter in placenta) and MXR (mitoxantrone resistance-associated protein), respectively. Molecular characterization revealed that BCRP consists of 655 amino acids with a molecular weight of 72.1 KD. In contrast to P-glycoprotein and MRP1 or MRP2, which contain a typical core structure of twelve transmembrane domains and two ATP binding sites, BCRP only has six transmembrane domains and one ATP binding site (Fig. 1), and therefore appears to be a half ABC transporter (230). BCRP is the second member of the ABCG subfamily containing members such as *drosophila white, brown and scarlet genes*, and thus "ABCG2" was recommended by the Human Genome Nomenclature Committee (HUGO) to refer to this newly identified transporter.

(3). As a half transporter, BCRP most likely forms a homodimer to transport its substrates out of the cells utilizing the energy derived from ATP hydrolysis (3, 229, 232-234). The murine homologue of BCRP, Bcrp1, has also been cloned and shown to be highly identical (81%) to BCRP with a virtually superimposable hydrophobicity profile (235). In addition, another gene closely related to Bcrp1 has also been identified in mice and named Bcrp2, which shares 54% identity with Bcrp1 (236). Whether Bcrp1 forms a heterodimer with Bcrp2 to perform its transport function remains unknown; however, the different expression patterns of these two genes indicate that Bcrp2 is not a necessary component for the transport function of Bcrp1. Distinct from other half transporters such as TAP1 and TAP2 (the transporters associated with antigen presentation), which are localized in the intracellular membranes (237), both human BCRP and murine Bcrp1 were shown to be predominantly present in the plasma membrane (16, 238, 239). Similar to P-glycoprotein and MRP1, both BCRP and Bcrp1 can be overexpressed *in vitro* upon drug selection or by transfection of cDNAs encoding these proteins, and confer multidrug resistance by the energy-dependent efflux of its substrates out of cells (229, 232, 233, 235, 240, 241). Significant and variable expressions of BCRP have been detected in human tumors such as acute leukemia and breast cancer; however, the contribution of this efflux transporter to the clinical MDR needs to be further investigated (242-248).

There is considerable overlap in the substrate specificity among P-glycoprotein, MRP1 or MRP2 and BCRP, although the binding affinity of a particular substrate to these transporters may vary substantially (3). The BCRP/Bcrp1 substrates identified so far include a number of anticancer agents such as anthracyclines (e.g., doxorubicin, daunorubicin, epirubicin), epipoxophyllotoxins (e.g., etoposide, teniposide), camptothecins or their active metabolites (e.g., topotecan, SN-38, 9-aminocamptothecin, CPT11), mitoxantrone, bisantrene, methotrexate, flavopiridol and HIV-1 nucleoside reverse transcriptase inhibitors (e.g., zidovudine, lamivudine); vincristine, paclitaxel and cisplatin appear not to be substrates (8, 229, 235, 249-253). The amino acid at the 482 position seems to be critical in defining substrate specificity because mutated forms of BCRP with arginine at the 482 position changed to threonine or glycine have shown different substrate preference

(251). Whether these mutated forms of BCRP also occur *in vivo*, especially in normal physiological situations, is currently unknown. However, similar phenomenon observed in mouse cell lines selected with doxorubicin, indicates that the 482 position appears to be a hot mutation spot and thus similar mutations might also happen in human tumors upon drug treatment (254). For investigating the pharmacological and physiological functions of BCRP and for MDR reversal, there is substantial effort in searching for and developing potent BCRP/Bcrp1 inhibitors. Fumitremorgin C (FTC) derived from *Aspergillus fumigatus* cultures appears to be the first identified potent and specific inhibitor for BCRP/Bcrp1 (255, 256); however, its *in vivo* application is limited by its neurotoxicity. The typical P-glycoprotein inhibitors, GF120918 and reserpine were also shown to be potent BCRP inhibitors (235, 257), but many of the other P-glycoprotein inhibitors such as LY335979, cyclosporin A, PSC833 and verapamil have little effect (8, 258). So far, the most potent specific BCRP inhibitor appears to be Ko134, an analogue of FTC (259). The compound has been used *in vivo* and demonstrated little or low toxicity in mice at high oral or i.p. doses, and could potentially be used *in vivo* for BCRP inhibition (259).

Interestingly, the distribution of BCRP in normal tissues is similar to P-glycoprotein. High levels of BCRP expression were detected in the human placenta syncytiotrophoblast plasma membrane, facing the maternal bloodstream, in the canalicular membrane of the liver hepatocytes, the apical membrane of the epithelium in the small and large intestine, in the ducts and lobules of the breast and in the luminal surface of brain capillaries (260, 261). In addition, significant amounts of BCRP were also found in venous, capillary, but not arterial, endothelial cells in almost all the tissues investigated (261). By analogy with P-glycoprotein, it is reasonable to speculate that one, if not the major physiological function of BCRP is to protect body or certain tissues from the exposure of toxic endogenous or exogenous compounds. The localization of BCRP in the placenta, brain and testis may regulate the entry of its substrates into the developing fetus, brain and other pharmacological sanctuaries, and therefore represents an important component of the blood-placenta, blood-brain and blood-testis barriers. The expression of BCRP in the luminal side of the intestinal epithelial cells and canalicular membrane of the hepatocytes suggests that the protein may play a significant role in intestinal secretion or back efflux.

to the intestinal lumen and in biliary excretion, thus limiting the entry of xenobiotic toxins into the systemic circulation or facilitating their elimination. A recent study conducted by Jonker et. al. (262), using Bcrp1 (-/-) knockout mice, strongly supports this speculation. In the study, the authors demonstrated that the oral bioavailability of topotecan increased about 6-fold in Bcrp1 (-/-) mice compared with the wild type mice and the accumulation of topotecan in Bcrp1 (-/-) fetuses was elevated 2-fold higher compared with the accumulation in the wild type fetuses (following normalization by the maternal plasma concentration), indicating Bcrp1 plays a critical role in the protection of the fetus from exposure to harmful substances. In addition, the authors also elegantly demonstrated that without functional Bcrp1, mice become at least 100-fold more sensitive to pheophorbide a, a dietary chlorophyll-breakdown product and Bcrp1 substrate, resulting in phototoxicity. The hypersensitivity could be explained by the markedly elevated plasma concentrations of pheophorbide a in these knockout mice, and therefore illustrates the importance of this transporter in the protection against natural toxins. Furthermore, studies from the same group also demonstrated that co-administration of topotecan with GF120918, a Bcrp1 inhibitor, dramatically increased the AUC of topotecan more than 6-fold due to the increased uptake from the intestine and the decreased biliary excretion in the *mdr1a* (-/-) mice (i.e., in the absence of P-glycoprotein) (Fig. 2) (16). Similar results have also been obtained when topotecan was co-administered with Ko134 (259). In humans, it has been shown that the apparent oral bioavailability of topotecan was significantly increased from 40.0% to 97.1% following the co-administration of GF120918. This change most likely resulted from the inhibition of BCRP; it is known that topotecan is only a weak substrate of P-glycoprotein (15). Taken together, there is convincing evidence that BCRP plays an important role in governing the body disposition of xenobiotics. It also should be noted that the expression of BCRP and MRP2 in the human intestine is even higher than P-glycoprotein (263), and therefore, it may be possible that the contribution of BCRP to the intestinal secretion and oral absorption of xenobiotics can be comparable with, if not greater than that of P-glycoprotein.

4. Other efflux transporters (MDR3, BSEP)

MDR3 is the other human P-glycoprotein isoform with virtually identical molecular structure to that of the human MDR1 and mouse *mdr1b* genes (46). MDR3 is mainly present in the canalicular membrane of liver hepatocytes and functions as an ATP-dependent phosphotidylcholine translocator (55-58). Initially, it was thought that MDR3 protein and its mouse homologue *mdr2* can not transport drugs and confer multidrug resistance (53, 54). But more recently, it has been shown that MDR3 is also capable of transporting several cytotoxic drugs such as digoxin, paclitaxel, and vinblastine, but with a low efficiency (264). A defect in MDR3 is believed to be associated with an autosomal recessive hereditary disorder, progressive familial intrahepatic cholestasis type 3 (PFIC3) (265, 266). BSEP (SPGP, ABCB11) is another homologue of MDR P-glycoproteins, initially identified from the pig and named the Sister of P-glycoprotein (spgp) (267). Subsequently, rat Bsep gene was also cloned and shown to be an ATP-dependent bile salt exporter with a K_m value of about 5 μM for transporting taurocholate (268). Bsep is almost exclusively present in the liver and localized to the canalicular microvilli and subcanalicular vesicles of the hepatocytes and functions as a major bile salt export pump in mammalian livers (267). The functional characterization of human BSEP has also been carried out recently and reported to have a similar K_m value for taurocholate (269, 270). A defect in BSEP in humans was associated with type 2 PFIC (PFIC2) (271-273). At this time, it is generally believed that both MDR3 and BSEP may not play a significant role in terms of drug disposition.

Conclusions

The molecular and functional characterization of efflux transporters, during the last 15 years, has facilitated our understanding concerning how these transporters control the passage of a diverse range of substrates through biological membranes. The characterization of their tissue localization and their function has suggested a significant impact of these transporters on the absorption, elimination and distribution of xenobiotic compounds as a body defense mechanism against the exposure of both endogenous and exogenous toxins. The generation of knockout mice lacking specific transporter(s) and the identification of specific inhibitors has greatly enhanced our ability to understand the physiological and pharmacological functions of these

transporters. It has been clearly demonstrated by the studies presented here, as well as others, that these efflux transporters play an essential role in intestinal absorption, biliary excretion, and renal secretion and contribute to the barrier functions between the blood and various tissues such as brain, testis and placenta. Considering the important impact of these efflux transporters in drug disposition, identification of substrates and inhibitors from commonly prescribed drugs or food-derived compounds and characterization of their kinetic parameters will help to predict potential drug interactions mediated by these transport mechanisms. However, the full appreciation of the impact of these transporters on drug disposition will depend on our understanding of the mechanism(s) by which these transporters recognize such a wide range of structurally distinct substances, the mechanism(s) by which these transporters are regulated, the influence of multiple co-existing transporters, as well as the interplay of these transporters with drug metabolizing enzymes; these aspects are all largely unknown at this time and remain to be investigated.

Acknowledgements

We acknowledge research support through the Susan G. Komen Breast cancer Foundation and U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376.

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Table 1. Characteristics of efflux transporters

| Member | HUGO symbol | Alternative name | Tissue localization | Subcellular level | Associated disease |
|--------|-------------|-------------------------|--|-------------------|------------------------|
| MDR1* | ABCB1 | PGY1, P-gp | liver, gut, kidney, adrenal gland, blood brain barrier, placenta | apical | drug resistance |
| MDR3* | ABCB4 | PGY3, MDR2/MDR3 | liver canalicular membrane | apical | PFIC3 |
| MRP1* | ABCC1 | MRP, GS-X | ubiquitous | basolateral | drug resistance ? |
| MRP2* | ABCC2 | cMOAT | liver, intestine, kidney | apical | Dubin-Johnson syndrome |
| MRP3* | ABCC3 | cMOAT2, MLP2, MOAT-D | liver, intestine, kidney, adrenal gland | basolateral | ? |
| BCRP* | ABCG2 | ABCP, MXR | placenta, liver, intestine apical membrane | apical | ? |
| BSEP** | ABCB11 | SPGP | liver canalicular membrane | apical | PFIC2 |

* Data from Litman et. al. (3).

** Data from Trauner and Boyer (274).

Table 2. Common substrates and inhibitors for the efflux transporters

| Transporter | Substrates | Inhibitors |
|-------------|--|---|
| MDR1 | anthracyclines, vinca alkaloids, epipodophyllotoxins, paclitaxel, topotecan*, mitoxantrone*, HIV protease inhibitors, digoxin, Rhodamine123, methotrexate | verapamil, diltiazem, trifluoperazine, quinidine, reserpine, cyclosporin A, valinomycin, terfenidine, PSC833, VX710*, PAK-104P, GF120918, LY35979*, XR9576* |
| MDR3 | phosphotidylcholine, digoxin (?), paclitaxel (?), vinblastine (?) | verapamil+, cyclosporin A+, and PSC833+ |
| MRP1 | Aflatoxin B1, doxorubicin, etoposide, vincristine, methotrexate, and various lipophilic glutathione, glucuronide and sulfate conjugates | MK571, cyclosporin A, VX710*, PA-104P* |
| MRP2 | glutathione conjugates, glucuronides, sulfate conjugates, methotrexate, temocaprilat, CPT11 arboxylate, SN38 carboxylate, cisplatin, pravastatin | MK571, cyclosporin A |
| MRP3 | glutathione conjugates, glucuronides, sulfate conjugates, methotrexate, monoanionic bile acids (taurocholate, glycocholate), vincristine, etoposide | MK571 (?) |
| BCRP | anthracyclines, epipoxophyllotoxins, camptothecins or their active metabolites, mitoxantrone, bisantrene, methotrexate, flavopiridol, zidovudine, lamivudine | FTC, GF120918, Ko-134 |
| BSEP | bile salts | |

Data were taken * (3) or + (263).

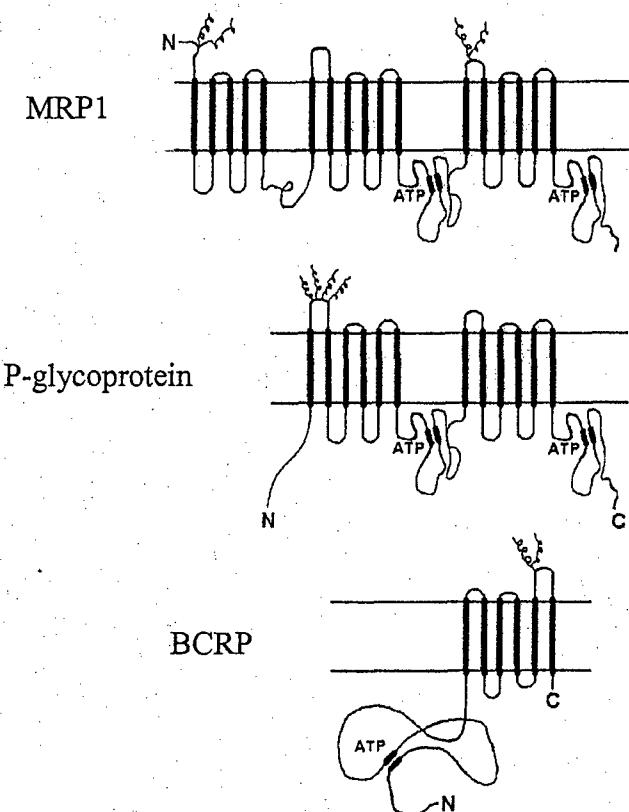


Fig. 1. Topology of efflux transporters MRP1, P-glycoprotein and BCRP
(Reproduced with permission from ref 3)

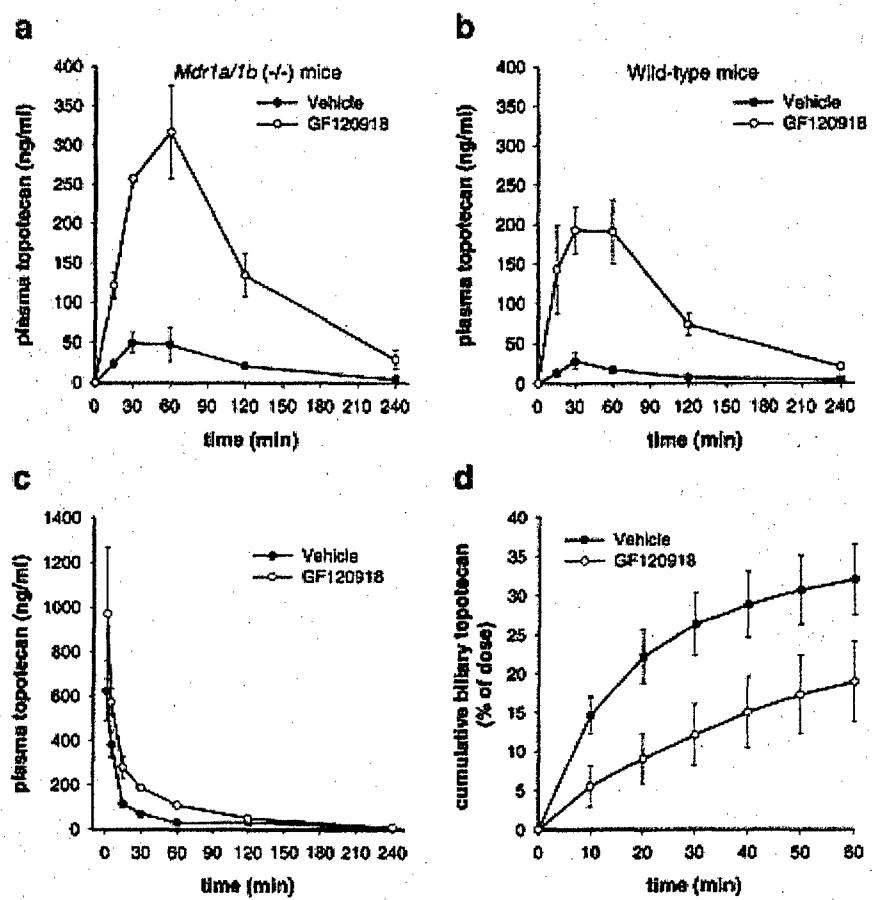


Fig. 2 Effects of GF120918 on the plasma concentration and biliary excretion of topotecan in mice

Mdr1a/1b (-/-) (a) or wild-type (b) mice were given an oral dose of GF120918 (50 mg/kg) or vehicle 15 minutes before an oral dose of topotecan (1 mg/kg). (c) *Mdr1a/1b* (-/-) mice were given an i.v. dose of topotecan in combination of an oral GF120918 or vehicle. (d) Cumulative biliary excretion of topotecan in *mdr1a/1b* (-/-) mice treated in the same way as (c). Results are the means \pm SD ($n \geq 3$). (Reproduced with permission from ref. 16).

proofs

Appendix 7

Dietary Organic Isothiocyanates Are Cytotoxic in Human Breast Cancer MCF-7 and Mammary Epithelial MCF-12A Cell Lines

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- ?1 Organic isothiocyanates (ITCs; i.e., mustard oils) are dietary components present in cruciferous vegetables. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC), β -phenethyl isothiocyanate (PEITC), and sulforaphane in human breast cancer MCF-7 and human mammary epithelium MCF-12A cell lines, as well as in a second human epithelial cell line, human kidney HK-2 cells. The cytotoxicity of NITC, BITC, PEITC, and sulforaphane, as well as the cytotoxicity of the chemotherapeutic agents daunomycin (DNM) and vinblastine (VBL), were examined in MCF-7/sensitive (wt), MCF-7/Adr (which overexpresses P-glycoprotein), MCF-12A, and HK-2 cells. Cell growth was determined by a sulforhodamine B assay. The IC₅₀ values for DNM and VBL in MCF-7/Adr cells were $7.12 \pm 0.42 \mu\text{M}$ and $0.106 \pm 0.004 \mu\text{M}$ (mean \pm SE) following a 48-hr exposure; IC₅₀ values for BITC, PEITC, NITC, and sulforaphane were 5.95 ± 0.10 , 7.32 ± 0.25 , 77.9 ± 8.03 , and $13.7 \pm 0.82 \mu\text{M}$, respectively, with similar values obtained in MCF-7/wt cells. Corresponding values for BITC, PEITC, NITC, and sulforaphane in MCF-12A cells were 8.07 ± 0.29 , 7.71 ± 0.07 , 33.6 ± 1.69 , and $40.5 \pm 1.25 \mu\text{M}$, respectively. BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells at concentrations similar to those of the chemotherapeutic drug DNM. Sulforaphane and NITC exhibited higher IC₅₀ values. The effect of these ITCs on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppressing the growth of preclinical tumors, and may indicate

a potential use of these compounds as chemotherapeutic agents in cancer treatment. *Exp Biol Med* 229:000–000, 2004.

Key words: phenethyl isothiocyanate; benzyl isothiocyanate; naphthyl isothiocyanate; sulforaphane; cytotoxicity

Organic isothiocyanates (ITCs) are plant-derived dietary compounds commonly known as mustard oils. Present in the *Brassica* genus of the Cruciferae family, they are found in vegetables such as cabbage, cauliflower, broccoli, and brussels sprouts, in the form of glucosinolates, the biosynthetic precursors of ITCs in plants. When vegetables are chewed, the glucosinolates are cleaved by the enzyme myrosinase (1) to form ITCs. Glucosinolate levels have been estimated to be as high as 180 mg/g in some vegetables (2). More than 20 natural and synthetic ITCs have demonstrated cancer-preventive properties in animals treated with chemical carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines (3–6). There is substantial evidence that inhibition of tumorigenesis is partly due to the direct inhibition or down-regulation (or both) of the cytochrome P-450 enzymes responsible for carcinogen activation, resulting in decreased amounts of ultimate carcinogens formed (7). In addition, ITCs have also demonstrated the ability to induce certain phase II enzymes, which are responsible for the detoxification of electrophilic intermediates formed during phase I metabolism (7). For example, ITCs can inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced carcinogenesis by inhibiting the microsomal metabolism of NNK to reactive species that form methyl and pyridyloxobutyl adducts in DNA (8). ITCs may also be able to suppress tumor cell growth. Recent studies have indicated that certain ITCs can affect the cell cycle; for example, sulforaphane induces cell cycle arrest and apoptosis in HT29 human colon cancer cells (9), PEITC also induces apoptosis *in vitro* (10, 11), and allyl ITC can induce apoptosis in dimethylhydrazine-induced rat colon cancer (12).

This research was supported in part by U.S. Army Contract DAMD17-00-1-0376 and by a grant from the Kapoor Charitable Foundation, SUNY. E.A.S.-R. was supported in part by a Gateway Research Scholarship from the American Foundation for Pharmaceutical Education.

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Received March 7, 2004.
Accepted May 4, 2004.

1535-3702/04/2295-0001\$15.00
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CYTOTOXICITY OF ISOTHIOCYANATES

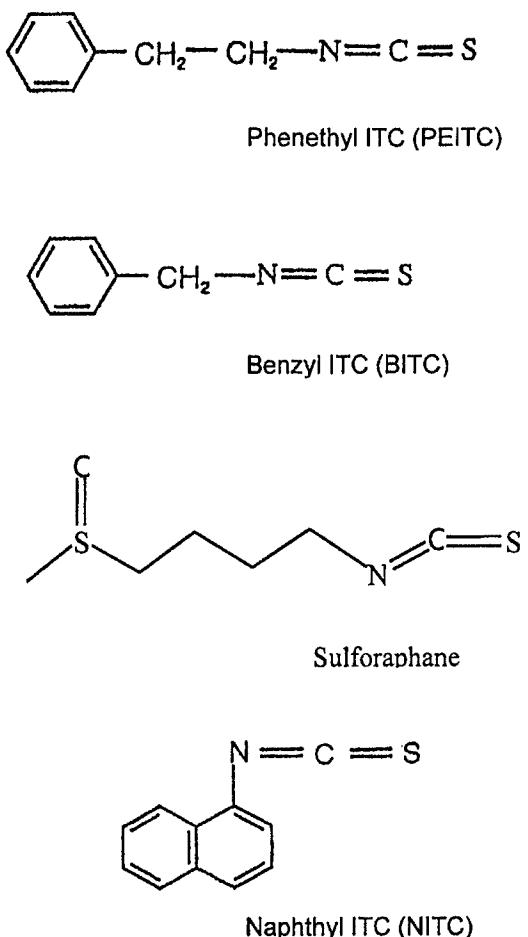


Figure 1. Chemical structures of phenethyl isothiocyanate, benzyl isothiocyanate, sulforaphane, and 1-naphthyl isothiocyanate.

The main objective of this investigation was to examine the cytotoxic effects of benzyl isothiocyanate (BITC), β -phenethyl isothiocyanate (PEITC), sulforaphane, and α -naphthyl isothiocyanate (NITC; Fig. 1) in a human breast cancer cell line (MCF-7) and a human mammary epithelium cell line (MCF-12A), and to compare the results with the cytotoxicity observed for two chemotherapeutic agents, daunomycin (DNM) and vinblastine (VBL). Cytotoxicities were also compared with those obtained using another human epithelial cell line, human kidney HK-2 cells.

Materials and Methods

Materials. PEITC, NITC, daunorubicin HCl, and vinblastine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). BITC was obtained from Aldrich (St. Louis, MO). Cell culture reagents were supplied by Gibco BRL (Buffalo, NY). Cell culture flasks and 96-well plates were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). The MCF-7/Adr cell line was obtained from the National Cancer Institute. The MCF-12A cell line was purchased from American Type Culture Collection (Manassas, VA). Epidermal growth factor (20 ng/ml) was obtained from BD Biosciences (Bedford, MA). Cholera

Table 1. IC_{50} Values of DNM and ITCs in MCF-7/Adr Cells

| Compound | Time (hours) | MCF-7/Adr IC_{50} Mean \pm SE, μM | MCF-7/sensitive IC_{50} Mean \pm SE, μM |
|--------------|-----------------|---|---|
| DNM | 2 | 40.1 \pm 1.74*** | — |
| | 4 | 34.2 \pm 1.32*** | — |
| | 6 | 21.9 \pm 2.47*** | — |
| | 48 | 7.12 \pm 0.42 | — |
| VBL | 2 | 9.99 \pm 0.803* | — |
| | 4 | 5.87 \pm 0.469 | — |
| | 6 | 6.99 \pm 0.787 | — |
| | 48 | 0.106 \pm 0.004 | — |
| BITC | 1 | 14.5 \pm 0.28*** | 17.0 \pm 1.53*** |
| | 2 | 10.4 \pm 0.31*** | 14.1 \pm 1.46*** |
| | 3 | 7.37 \pm 0.16*** | 10.3 \pm 1.62*** |
| | 6 | 5.55 \pm 0.19*** | 6.94 \pm 0.46* |
| | 48 | 5.95 \pm 0.10 | 4.11 \pm 0.26 |
| | 1 | 20.4 \pm 0.78*** | 30.3 \pm 0.98*** |
| | 2 | 14.0 \pm 0.54*** | 20.8 \pm 1.95*** |
| | 3 | 12.8 \pm 0.65*** | 15.2 \pm 0.10* |
| PEITC | 6 | 7.56 \pm 0.31*** | 22.6 \pm 3.54* |
| | 48 | 7.32 \pm 0.25 | 6.51 \pm 0.86 |
| | 3 | — | 89.2 \pm 10.8 |
| | 48 | 77.9 \pm 8.03 | 78.4 \pm 5.26 |
| Sulforaphane | 3 | 167 \pm 3.61*** | 144 \pm 26.1*** |
| | 6 | 64.1 \pm 3.37 | 119 \pm 12.4*** |
| | 48 | 13.7 \pm 0.82 | 27.9 \pm 5.30 |
| | — | — | — |

Significantly different from 48-hr values: * $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$; n = 2–8.

toxin (100 ng/ml), insulin (10 $\mu\text{g}/\text{ml}$), and hydrocortisone (500 ng/ml) were purchased from Sigma Chemical.

Cell Culture. MCF-7/wt and MCF-7/Adr cells (passages 16–24) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (10 units/ml), and streptomycin (10 $\mu\text{g}/\text{ml}$). Cells were incubated at 37°C supplemented with 5% CO₂/95% air. Cells were subcultured two to three times a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 75-mm² plastic culture flasks and seeded in 96-well plates.

MCF-12A cells, used between passages 56–58, were grown in a 1:1 mixture of Dulbecco modified Eagle medium and Ham's F12 medium supplemented (95%) with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 $\mu\text{g}/\text{ml}$ insulin, and 500 ng/ml hydrocortisone and 5% horse serum. Cells were incubated at 37°C supplemented with 5% CO₂/95% air. Cells were subcultured using 0.25% trypsin-0.03% EDTA in phosphate-buffered saline (PBS). Cells were grown in 75-mm² plastic culture flasks and seeded in 96-well plates.

HK-2 cells, used between passages 15–25, were grown in Keratinocyte-SFM containing 5 ng/ml recombinant epidermal growth factor and 0.04 mg/ml bovine pituitary extract. Cells were incubated at 37°C supplemented with 5% CO₂/95% air. Cells were subcultured once a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 75-mm² plastic culture flasks and seeded in 96-well plates.

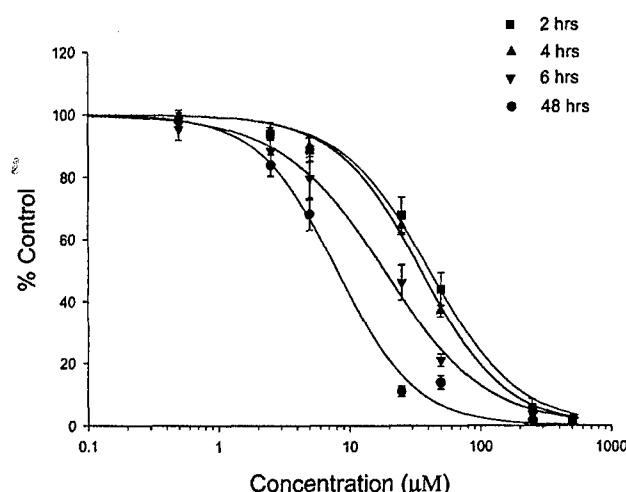


Figure 2. DNM cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of DNM on cell growth of MCF-7/Adr cells following exposure times of (■) 2 hrs, (▲) 4 hrs, (▼) 6 hrs, and (●) 48 hrs. Each data point represents the mean \pm SE from four wells in one representative study. The study was repeated five to eight times.

Cytotoxicity Assay. Cells were plated at a density of 5,000 cells/well for 48 hrs prior to drug exposure. The doubling rate for MCF-7 cells is \sim 28 hrs; therefore, plating cells for 48 hrs makes certain that cells are in their exponential phase when exposed to drugs. Cells were then exposed to 200 μ l of growth media (containing 0.5% dimethyl sulfoxide; DMSO) in the absence or presence of increasing concentrations of NITC, BITC, PEITC, sulforaphane, DNM, or VBL for time intervals up to 48 hrs. At the end of the incubation period, the cells were rinsed twice with 1X PBS, and fresh media was added. Control wells were incubated with the vehicle DMSO; the percentage of DMSO in media was kept constant (0.5%); a concentration that demonstrated no cytotoxicity in any cell line. At 48 hrs, media was removed and a sulforhodamine B (SRB) assay was performed (13). Briefly, cells were fixed to the plate with 100 μ l of 10% trichloroacetic acid and stored at 4°C for 1 hr. Cells were then rinsed five times with water and allowed to dry for 5–10 mins. A 100- μ l aliquot of SRB 0.4% dye was added to each well for 15 mins and the wells were rinsed 3–4 times with 1% acetic acid. Plates were allowed to air dry for another 5–10 mins before the addition of 10 mM Tris-base for 5 mins. Absorbance was read at 570 nM, with a Spectra Rainbow plate reader (Tecan US; SLT Lab Instruments, Research Triangle Park, NC). The absorbance values (OD 570) from the SRB assay indicate the cell number in each well of the 96-well plates.

Data Analysis. Data were fitted to a sigmoidal E_{max} equation using WinNonlin pharmacokinetic software (Pharsight Corp., Mountain View, CA):

$$E = E_0 - \left(\frac{E_{max} \times C^\gamma}{IC_{50}^\gamma + C^\gamma} \right)$$

where E represents the effect, E_0 is the initial effect, E_{max} is the maximal effect, C is the concentration of substrate, IC_{50}

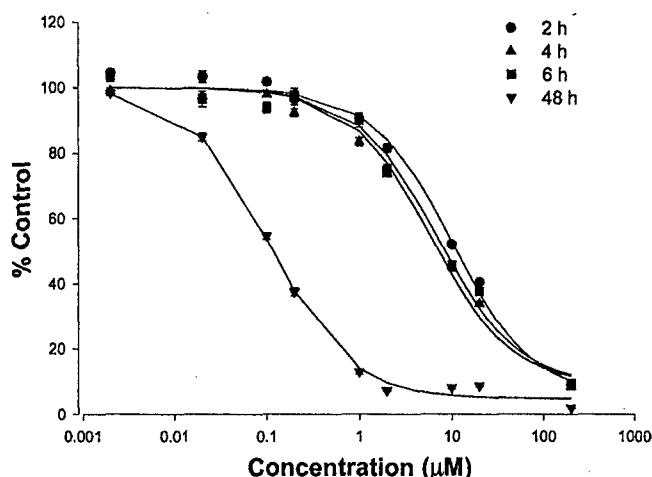


Figure 3. VBL cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of VBL on cell growth of MCF-7/Adr cells following exposure times of (●) 2 h, (▲) 4 h, (■) 6 h, and (▼) 48 h. Each data point represents the mean \pm SE from four wells in one representative study. The study was repeated twice.

is the concentration that inhibits cell growth by 50%, and γ represents a sigmoidicity factor. Statistical analysis was determined by a one-way ANOVA followed by a Dunnett posthoc test using the analysis software GraphPad Prism (GraphPad Inc., San Diego, CA). Differences were considered to be significant when $P < 0.05$.

Results

Cytotoxicity as a Function of Time Exposure.

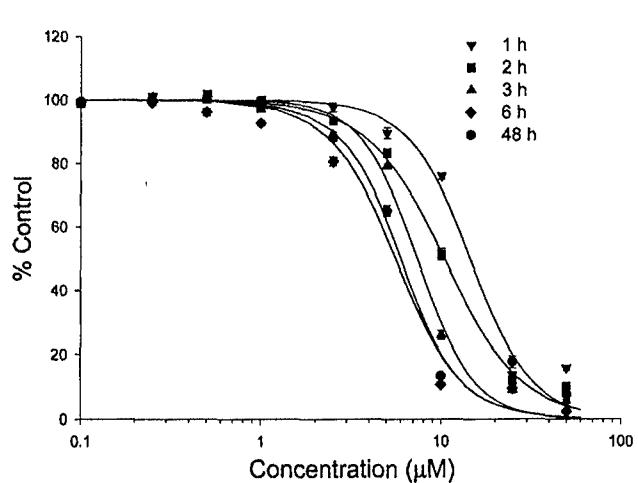
The general pattern observed in the MCF-7 cell lines was a decrease in IC_{50} values with longer exposure times to the test drugs (Table 1). The IC_{50} values for DNM and VBL decreased significantly over the 48-hr exposure period, with the lowest values obtained following a 48-hr exposure.

Cytotoxicity Studies with MCF-7 Cells. The IC_{50} value of DNM in MCF-7/Adr cells determined following a 48-hr exposure was $7.12 \pm 0.42 \mu\text{M}$, while that of VBL was $0.106 \pm 0.004 \mu\text{M}$. The general potency, determined after a 48-hr exposure, was VBL > BITC PEITC DNM > sulforaphane > NITC (Table 1; Figs. 2–4). In MCF-7/Adr cells, IC_{50} values for the ITCs were generally similar to those in MCF-7/wt and MCF-12A cells (except for NITC in MCF-12A cells). PEITC and BITC were more cytotoxic than DNM after an exposure of 6 hrs and comparable after a 48-hr exposure.

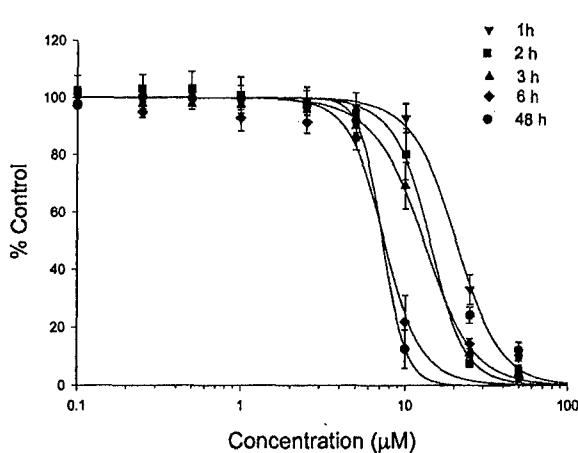
Cytotoxicity Studies with MCF-12A Cells. The IC_{50} value of DNM was not determined at concentrations up to $100 \mu\text{M}$ following a 48-hr exposure time in MCF-12A cells. When BITC, NITC, PEITC, and sulforaphane were incubated with MCF-12A cells *in vitro*, there was a dose-dependent inhibition of cell growth (Table 2; Fig. 5). BITC and PEITC inhibited the growth of MCF-12A cells with similar potency; IC_{50} values ranged from 7.71 to 8.07 μM for a 48-hr exposure. Sulforaphane inhibited cell growth with a similar IC_{50} value as that in MCF-7 cell lines. NITC

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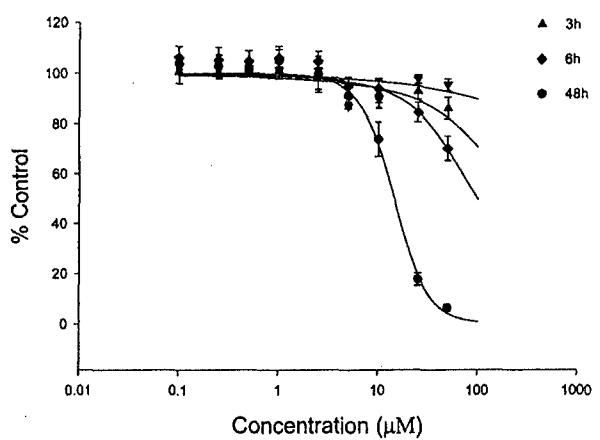
A. BITC



B. PEITC



C. Sulforaphane



D. NITC

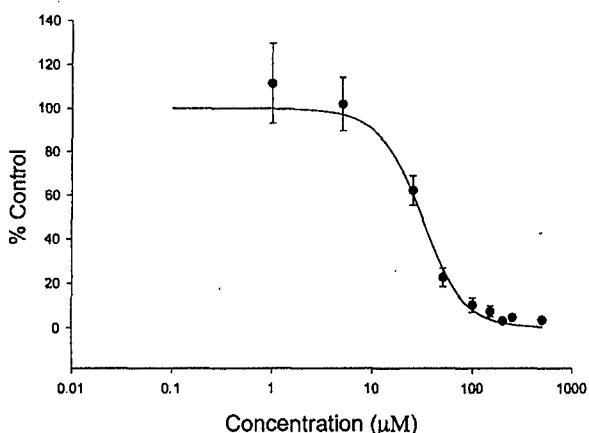


Figure 4. ITC cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of (A) BITC, (B) PEITC, (C) sulforaphane, and (D) NITC on cell growth of MCF-7/Adr cells following exposure times of (▼) 1 hr, (■) 2 hrs, (▲) 3 hrs, (♦) 6 hrs, and (●) 48 hrs. Each data point represents the mean \pm SE from four wells in one representative study. The study was repeated two to four times.

exhibited less toxicity than BITC and PEITC, although the IC₅₀ value was about one-half that of the MCF-7 cell lines.

Cytotoxicity Studies with HK-2 cells. DNM was observed to have a mean IC₅₀ of 0.77 μ M following a 48-hr exposure in HK-2 cells. When BITC, PEITC, and NITC were incubated with HK-2 cells *in vitro*, all produced inhibition of cell growth (Table 3). BITC and PEITC inhibited the growth of HK-2 cells with similar potency: IC₅₀ values ranged from 2.12 to 2.62 μ M for a 2-hr exposure and from 2.97 to 3.27 μ M for a 48-hr exposure. However, NITC exhibited less toxicity than BITC and PEITC. The IC₅₀ values for NITC averaged 10.73 μ M for a 2-hr exposure and 7.26 μ M for a 48-hr exposure.

Discussion

The capacity for organic ITCs to block chemical carcinogenesis was first recognized more than 30 years ago with NITC. ITCs have been demonstrated to be potent chemopreventive agents in numerous animal models of cancer (5, 14–16). The inhibition of carcinogen metabolic activation via inhibition of cytochrome P-450 enzymes and increased detoxification of carcinogens through induction of glutathione S-transferases (GSTs) and UDP-glucuronyl transferases (UGT) or NADPH quinone oxidoreductase (NQO1) are recognized as important mechanisms (17). Human and rodent studies have demonstrated that PEITC blocks metabolic activation of NNK and benzo(a)pyrene, major lung carcinogens in tobacco smoke, via CYPs, 25

Table 2. IC₅₀ Values of ITCs in MCF-12A Cells

| Compound | Time (hours) | IC ₅₀ Mean ± SE μM (n = 4) |
|--------------|--------------|--|
| BITC | 48 | 8.07 ± 0.29 |
| PEITC | 48 | 7.71 ± 0.07 |
| NITC | 48 | 33.6 ± 1.69 |
| Sulforaphane | 48 | 40.5 ± 1.25 |

resulting in increased urinary excretion of detoxified metabolites, and suggesting inhibitory effects on CYP1A1, CYP1A2, and CYP2B1 (8, 18, 19). In humans, watercress ingestion resulted in a reduction in the levels of oxidative metabolites of acetaminophen, which was attributed to inhibition of oxidative metabolism by CYP2E1 (20), and enhancement in the area under the plasma concentration-time curve of chlorzoxazone, a clinical probe for CYP2E1 (21). ITCs are able to increase the expression or activity (or both) of phase II enzymes in rat organs (7) and in cell culture (22). Watercress extract increases the activities of UGT, GST, and NQO1 (23). PEITC treatment of LS-174 human colon cells produced an increased protein expression of NQO1 and γ-glutamylcysteine synthetase (24), and PEITC, administered to rats by gavage or via diet, induces hepatic NQO1, GST, and UGT (18, 25).

In this investigation we found that BITC and PEITC inhibit cell growth in breast cancer cells at similar concentrations as those observed for DNM. These concentrations are 4- to 6-fold lower than the IC₅₀ for the isoflavonoid genistein, a compound that has also been studied in MCF-7 cells. Genistein has been reported to have the lowest IC₅₀ value among the dietary flavonoids tested in MCF-7 cells (26). Although the plasma concentrations of ITCs following dietary consumption in humans is unknown, it has been estimated that the consumption of 100 g of broccoli could release 40 μmol of the ITC sulforaphane, which would result in low micromolar concentrations in plasma (27). PEITC is a naturally occurring ITC, being found as its glucosinolate conjugate gluconasturtiin in vegetables, including watercress. Consumption of about 30 g of watercress releases about 46.5 μmol of PEITC (28). Another factor that will highly influence plasma ITC levels is the polymorphic expression of the major metabolizing enzymes for many ITCs, GSTM1 and GSTT1 (29). Polymorphisms in the GSTM1 and GSTT1 genes are caused by a complete deletion of the gene, which results in loss of function. The incidence of homozygous null deletion is approximately 50% for GSTM1 in white subjects in the United States, as well as in Japanese and Chinese subjects; for GSTT1 the incidence is 12%-16% in subjects from Germany and England, and 60-64% in subjects from China and Korea (30). Moreover, the protective effect of broccoli on the prevalence of colorectal adenomas was observed only among subjects with the GSTM1-null genotype (31). Studies examining the correlation of ITC intake obtained

through vegetable consumption and GSTM1 and GSTT1 genotypes among various populations have also suggested that the reduction in lung cancer risk was stronger among persons genetically deficient in GSTM1, GSTT1, or both, although GSTM1 deficiency appears to be more important (32-34). In those subjects with detectable levels of ITCs in their urine and a GSTM1 deficiency, there was a 64% decrease in the risk for developing lung cancer (32). These studies suggest the importance of the polymorphic expression of GSTs in determining the systemic concentrations and efficacy of ITCs.

The mechanism underlying the cytotoxicity of ITCs is unknown, but apoptosis may play an important role. PEITC is capable of inducing apoptosis in a dose-dependant manner in HeLa cervical cancer cells, human leukemia HL-60 cells, PC-3 prostate cancer cells, and in human HT-29 colon carcinoma cells (11). In T-29 colon adenocarcinoma cells and HT-29 cells, PEITC induces apoptosis via the mitochondria caspase cascade and the activation of c-Jun N-terminal kinase (JNK) is critical in this process. This leads to cytochrome *c* release, activation of caspase-9 and caspase-3, followed by nuclear condensation and DNA fragmentation (11). The tumor suppressor protein p53 is involved in ITC-mediated apoptosis but is not essential, because apoptosis can also occur in p53-null cells. Additionally, the PEITC-induced apoptosis in p53-deficient PC-3 prostate cancer cells is mediated by extracellular-regulated kinase and not JNK, suggesting cell line dependence. Thornalley (22) has suggested that depletion of glutathione due to the formation and subsequent expulsion of glutathione conjugates from cells may result in protein thiocarbamoylation by the ITC. Protein thiocarbamoylation may lead to the activation of JNK, with the subsequent induction of apoptosis (22). BITC and PEITC can deplete intracellular GSH concentrations in cell lines, whereas NITC does not (35). NITC differs from PEITC and BITC in that it is not a naturally occurring ITC and it is not metabolized by GST enzymes. While there are no reported cellular toxicities of NITC, there have been reported cases of hepatotoxicity by this compound. Primary rat hepatocyte cultures incubated for 18 hrs with NITC (0-100 μM) produced cytotoxicity at concentrations >25 μM (36). Therefore, the mechanisms involved in the cytotoxicity of NITC may differ from that of BITC and PEITC.

There are a number of possible reasons for the differences in IC₅₀ values observed in this study in MCF-7/Adr, MCF-12A, and HK-2 cells, besides differences in intrinsic sensitivity to the ITCs. The human breast cancer cell line MCF-7/Adr has pronounced expression of P-glycoprotein, which may result in lower intracellular concentrations of the ITCs in MCF-7/Adr cells if the ITCs are substrates for P-glycoprotein. However, we have shown that PEITC is not a substrate for P-glycoprotein in MCF-7/Adr cells, and that intracellular concentrations are similar in sensitive and resistant MCF-7 cells (35). Additionally, in this study, the IC₅₀ values of the ITCs were similar in MCF-

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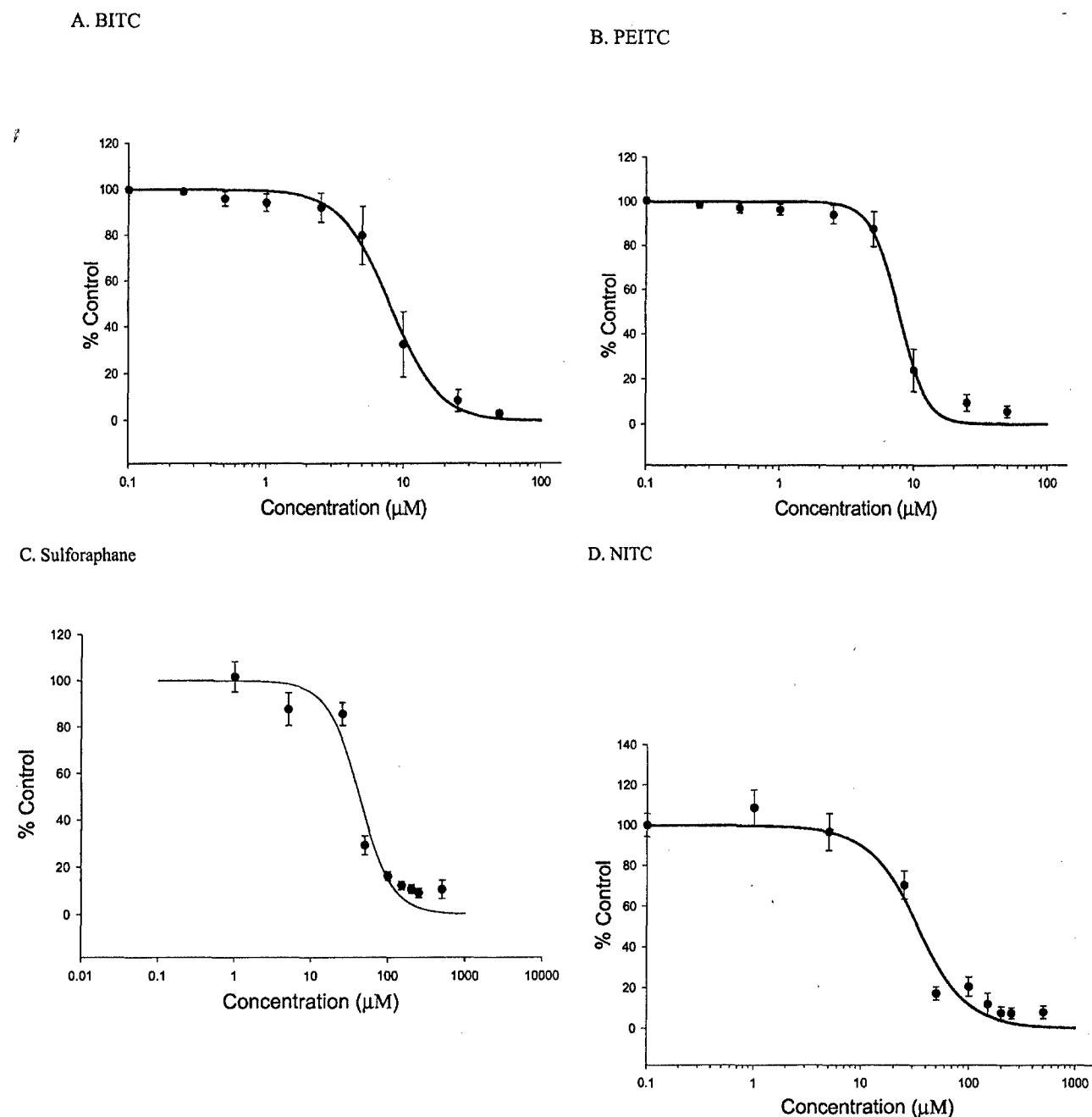


Figure 5. ITC cytotoxicity in MCF-12A cells. The effect of varying concentrations of (A) BITC, (B) PEITC, (C) sulforaphane, and (D) NITC on cell growth of MCF-12A cells following exposure time of (●) 48 hrs. Each data point represents the mean \pm SE from four wells in one representative study. The study was repeated 4 to 10 times.

7/wt and MCF-7/Adr cells, indicating that the presence of P-glycoprotein did not influence efficacy. Differences in intracellular concentrations in cell lines may also occur due to differences in protein binding of the ITC in the cell media. In a study by Xu and Thornalley (37), it was found that in the absence of FBS, the IC_{50} of PEITC in HL-60 cells was lower compared with the increased IC_{50} values in the presence of 10% and 25% FBS in the media. The MCF-7/Adr media used in this investigation contained 10% FBS and the MCF-12A media contained 5% horse serum, whereas the HK-2 media did not contain protein. If the

ITCs are protein-bound, then lower free concentrations would be present in protein-containing media, and would therefore be available to diffuse across cell membranes in MCF-7 cells. Preliminary studies in our laboratory have indicated extensive protein binding of PEITC in rat serum (Y. Kuo and M. E. Morris, unpublished results). The lack of protein binding in HK-2 cell media may be responsible for the lower IC_{50} values for ITCs observed in the HK-2 cell studies. Intracellular levels of ITCs will also be dependent on drug metabolism within these cells and on stability of the ITC in the culture medium, which may differ substantially.

Table 3. IC₅₀ Values of DNM and ITCs in Human Kidney Proximal Tubule HK-2 Cells

| Compound | Time (hours) | IC ₅₀ Mean ± SE (μM) |
|----------|--------------|------------------------------------|
| DNM | 48 | 0.768 ± 0.060 |
| BITC | 2 | 2.19 ± 0.04 |
| | 6 | 1.47 ± 0.03*** |
| PEITC | 48 | 2.97 ± 0.05 |
| | 2 | 2.62 ± 0.07*** |
| | 6 | 2.35 ± 0.06*** |
| NITC | 48 | 3.27 ± 0.04 |
| | 2 | 10.7 ± 0.41*** |
| | 6 | 5.69 ± 0.29** |
| | 48 | 7.26 ± 0.09 |

Significantly different from 48-hr values: *P < 0.01, **P < 0.05, ***P < 0.001; n = 4–8.

The intracellular accumulation of some ITCs has been shown to be highly dependent on intracellular glutathione concentrations; differences in GSH and GST activity in the two cell lines could result in different degrees of accumulation at the same media ITC concentration (38, 39).

The IC₅₀ values for the ITCs remained the same in both MCF-7/Adr and MCF-12A cells following 6- and 48-hr exposure times. This contrasted with our findings with DNM and VBL, in which the IC₅₀ values were significantly lower following a 48-hr exposure. Although the reason for this difference is unknown, this may be due to the stability and metabolism of ITCs at 37°C in these cell lines. We have found that the half-life for PEITC in buffer at pH 7.4 at room temperature is about 58 hrs, while that of BITC is about 40 hrs; NITC is less stable in buffer, with a half-life of approximately 20–25 hrs (Y. Kuo and M. E. Morris, unpublished). Metabolism in these cell lines for any of the tested compounds has not been examined. As well, adsorption to plastic wells for these very lipophilic compounds may represent a source of loss of the ITCs (35).

In conclusion, we have shown that the ITCs BITC, PEITC, NITC, and sulforaphane are able to exert cytotoxic effects in a dose- and time-dependent manner. This cytotoxic effect, which is comparable for BITC and PEITC with that of DNM, may represent another mechanism that is important for the chemopreventive effect of the ITCs, or it may indicate the potential use of BITC and PEITC as anticancer agents. Elucidation of the mechanisms underlying the cancer-protective effects of ITCs is of crucial importance, not only for the use of these compounds as chemopreventive agents, but also for the identification or design of more effective chemopreventive agents.

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*submitted - under revision
(minor revision requested)*

Appendix 8

Effect of Organic Isothiocyanates on Breast Cancer Resistance Protein (ABCG2)-

Mediated Transport

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Running title: Organic Isothiocyanates and BCRP

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ABSTRACT

Purpose. To investigate the effect of organic isothiocyanates (ITCs), dietary compounds with chemopreventive activity, on breast cancer resistance protein (BCRP)-mediated transport.

Methods. The effect of twelve ITCs on the cellular accumulation of mitoxantrone (MX) was measured in both BCRP-overexpressing and BCRP-negative human breast cancer (MCF-7) and large cell lung carcinoma (NCI-H460) cells by flow cytometric analysis. The ITCs showing activity in MX accumulation were examined for their effect on MX cytotoxicity and the intracellular accumulation of radiolabeled PEITC was measured in both BCRP-overexpressing and BCRP-negative NCI-H460 cells.

Results. ITCs significantly increased MX accumulation and reversed its cytotoxicity in resistant cells, but had a small or no effect in sensitive cells. The effects of ITCs on MX accumulation and cytotoxicity were ITC-concentration dependent. Significant increases in MX accumulation were observed at ITC concentrations of 10 or 30 μ M and significant reversal of MX cytotoxicity was generally observed at ITC concentrations of 10 μ M. Intracellular accumulation of radiolabeled PEITC in BCRP-overexpressing cells was significantly lower than that in BCRP-negative cells, and was increased significantly by the BCRP inhibitor fumitremorgin C (FTC).

Conclusions. Certain ITCs are BCRP inhibitors and PEITC and/or its cellular metabolite(s) may represent BCRP substrates, suggesting the potential for diet-drug interactions.

KEY WORDS: breast cancer resistance protein, isothiocyanates, membrane transport

INTRODUCTION

Transmembrane transporters of the ATP-binding cassette (ABC) superfamily act as efflux pumps and result in decreased intracellular concentrations of various structurally and functionally unrelated compounds. Often overexpressed in tumor cells, these transporters actively export anticancer drugs, decreasing intracellular concentrations below therapeutic thresholds and conferring multidrug resistance (MDR). ABC transporters are also present in many normal tissues such as intestine, blood brain barrier, liver and kidney, where they serve a protective role, through the ATP-dependent efflux of xenobiotics and toxins. As a consequence, ABC transporters play an important role in drug absorption, disposition and elimination (1).

Breast cancer resistance protein (BCRP), also known as ABCG2, mitoxantrone resistant protein (MXR) or ABC transporter in placenta (ABCP), is a half-size ABC transporter with only one N-terminal ATP binding domain and one C-terminal transmembrane region with six transmembrane segments (2). BCRP mediates resistance to chemotherapeutic agents including mitoxantrone (MX), topotecan, doxorubicin (DOX) and SN-38 (the active metabolite of CPT-11) (3). BCRP also transports sterols, steroids and estrogen sulfate conjugates, suggesting its physiological role in humans (4,5). Localized in the plasma membrane, BCRP is widely present in human tumor tissues and the BCRP overexpression has been associated with poor responses in clinical chemotherapy (6,7). BCRP is also expressed in a wide variety of normal tissues including placenta, liver, intestine, colon, lung, kidney, adrenal, sweat glands, and the endothelia of veins and capillaries (2). Localized in the apical membranes of intestinal epithelium and the biliary canalicular membranes, BCRP affects the intestinal uptake of orally administered drugs and their hepatobiliary excretion (3). In fact, BCRP and Multidrug Resistance-associated Protein 2 (MRP2) transcripts are more abundant than MDR1 (for P-glycoprotein) or MRP1 transcripts

in normal human jejunum (8). Clinical studies have demonstrated that inhibition of intestinal BCRP improves the oral bioavailability of topotecan in humans (9); and mice lacking Bcrp1/Abcg2 were reported to be extremely sensitive to diet-induced phototoxicity by pheophorbide a, suggesting a greater bioavailability of pheophorbide a in bcrp1-deficient mice (10).

Organic isothiocyanates (ITCs) ($R-N=C=S$) occur in cruciferous vegetables as glucosinolates, which can be hydrolyzed to ITCs by myrosinase released from damaged plant cells. ITCs are of interest since numerous studies have demonstrated that more than 25 natural and synthetic ITCs block the carcinogenic effects of a variety of chemically different types of carcinogens in over 10 target sites (11). Human glucosinolate consumption has been assessed to be as high as 300 mg/d (11) and consuming normal amounts of vegetables such as watercress or broccoli releases milligram amounts of ITCs (12). Besides the food sources, health products containing ITCs as food supplements have emerged in the market recently, as the result of increasing public attention to the health beneficial property of ITCs. Accordingly, high ITC concentrations in the intestine may be achieved, and one may anticipate food-drug interactions.

Our laboratory has demonstrated that certain ITCs are weak inhibitors of P-glycoprotein (P-gp)- and MRP1-mediated transport (13). Whether ITCs have effects on BCRP, which may be important in both MDR and intestinal drug absorption, is not known. Therefore, the present study was designed to examine the effect of ITCs on BCRP-mediated transport. Twelve dietary and synthetic ITCs were evaluated for their effects on the cellular accumulation and cytotoxicity of MX in both BCRP-overexpressing and BCRP-negative cells. The compounds evaluated were allyl- (AITC), benzyl- (BITC), hexyl- (HITC), 1-naphthyl- (NITC), phenyl- (PITC), phenethyl- (PEITC), phenylhexyl- (PHITC), phenylpropyl- (PPITC), phenylbutyl-isothiocyanate (PBITC), sulforaphane, erucin and erysolin. The intracellular

accumulation of radiolabeled PEITC was also measured in both BCRP-overexpressing and BCRP-negative cells, to determine if PEITC is a substrate for BCRP.

MATERIALS AND METHODS

Materials

AITC, BITC, HITC, NITC, PITC, PEITC, erysolin, MX and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO). Sulforaphane, PBITC and PPITC were purchased from LKT Laboratories (St. Paul, MN). Erucin was purchased from ICN Biomedicals, Inc. (Irvine, CA). PHITC was a gift from National Cancer Institute-Chemopreventive Division (Bethesda, MD). GF120918 was obtained from GlaxoSmithKline Inc. (Research Triangle Park, NC). Doxorubicin hydrochloride (DOX) was purchased from Sicor Pharmaceuticals, Inc. (Irvine, CA). Radiolabelled ¹⁴C-PEITC (specific activity 8.1 mCi/mmol, chemical purity > 97% by HPLC) was synthesized and characterized by Qi Wang in our laboratory. RPMI 1640, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were supplied by Gibco BRL (Buffalo, NY). MCF-7/sensitive and the resistant MCF-7/MX100 cells, NCI-H460 and the resistant NCI-H460/MX20 cells, as well as fumitremorgin C (FTC) were kindly provided by Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). Biodegradable liquid scintillation cocktail was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture

MCF-7/sensitive, MCF-7/MX100, NCI-H460, and NCI-H460/MX20 cells were grown in RPMI 1640 supplemented with 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin. MX of 100 and 20 nM were supplemented for MCF-7/MX100 and NCI-H460/MX20 cells, respectively. Expression of BCRP was confirmed by Western blot analysis as described previously (14). Cells were incubated in 75 mm² plastic culture flasks at 37°C supplemented with 5% CO₂/95% air.

MX Accumulation Assay

The MX accumulation assay was performed as described previously (14). Briefly, after being trypsinized and washed with FBS-free RPMI 1640, 1 ml of cells ($\sim 10^6$ cells) were exposed with various concentrations of ITCs, the vehicle (0.1% DMSO), or 10 μM FTC (as a positive control) at 37°C for 15 min, followed by the addition of 3 μM of MX for 30 min.

The accumulation was stopped by adding 3 ml of ice-cold PBS, followed by centrifugation. After cells were washed with ice-cold PBS two more times, the intracellular level of MX was determined by measuring MX fluorescence using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a standard argon laser for 488-nm excitation and a 670 nm bandpass filter. The accumulation of MX was expressed as percent of the control, where control represented cells treated with the vehicle.

MX Cytotoxicity Assay

The cytotoxicity assay was performed as described previously with minor modifications (14). Briefly, cells were trypsinized and plated in 96-well plates at a density of 5,000 (for NCI-H460) or 10,000 (for NCI-H460/MX20) cells per well. After 48-h attachment, fresh medium containing a serial dilution of MX as well as the specified concentrations of ITCs, the vehicle (0.1% DMSO), or 10 μM FTC (as a positive control), was added to the plates. The MX concentration range used for NCI-H460 and NCI-H460/MX20 cells were 0-300 and 0-1000 nM, respectively. Cells were incubated for another 24 hours and cell growth in each well was determined by a SRB assay (15). The absorbance values at 570 nm (OD_{570}) from the SRB assay represent the cell number in each well of the plates. The IC_{50} value, the concentration required to inhibit 50% of cell growth, was determined by fitting cell growth curves using WinNonlin Professional 2.1 (Pharsight, Mountain View, CA). The fitting

equation was $F = 100 \times \left(1 - \frac{I_{\max} \cdot C'}{IC'_{50} + C'}\right)$, where C is the concentration of MX and F is cell

survival fraction. F was calculated as 100 times the ratio of the cell growth ($OD_{570} - OD_{570}(\text{maximal concentration})$) to the maximum cell growth ($OD_{570}(0) - OD_{570}(\text{maximal concentration})$), where $OD_{570}(0)$ and $OD_{570}(\text{maximal concentration})$ are the absorbance values from cells treated without and with the maximal concentration of MX, respectively. Quadruplicate measurements were performed in each experiment.

¹⁴C-PEITC Accumulation Assay

The accumulation of ¹⁴C-PEITC was evaluated as previously described with minor modifications (13). Cells were trypsinized and seeded on 35 mm² dishes at a density of 5×10^5 cells per dish and used two days later. Growth medium was removed from monolayer cells and cells were washed twice with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, pH 7.4). One ml of sodium buffer containing specified concentrations of ¹⁴C-PEITC (supplemented with cold PEITC) with or without other modulators was added to the dish and incubated for 1 hour. FTC and GF120918, inhibitors of BCRP, were used as positive controls. The accumulation was stopped by aspirating the incubation buffer and washing the cells three times with ice-cold stop solution (137 mM NaCl, 14 mM Tris-base, pH 7.4). One ml 0.3 N NaOH-1% SDS was added to each dish, and cell lysates were collected after an hour. The radioactivity was measured by a liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co.) and the protein concentration was determined by a BCA assay using a commercially available assay kit (Pierce Biotechnology, Inc., Rockford, IL) (16).

Statistical Analysis

Statistical evaluation was performed using a one-way ANOVA followed by Dunnett's post hoc test or using a student's t-test. Significant differences were considered when $p < 0.05$.

RESULTS

Effect of ITCs on the Intracellular Accumulation of MX

MX is a specific substrate for BCRP with high binding affinity. To examine whether organic ITCs modulate BCRP, a MX accumulation study was performed in BCRP-overexpressing cells (MCF-7/MX100 and NCI-H460/MX20 cells) as well as the corresponding BCRP-negative cells (MCF-7/sensitive and NCI-H460 cells). The four cell lines have been characterized in our laboratory previously (14). FTC, a specific BCRP inhibitor, was used as a positive control in the study. The fluorescence values of all the tested ITCs at the settings used in the study are negligible (data not shown). After cells were exposed to 50 μ M ITCs and 3 μ M MX, intracellular accumulation of MX in MCF-7/MX100 cells was significantly increased by BITC, PEITC, NITC, PBITC, PPITC, HITC and PHITC by 3.5- to 5.8-fold ($p < 0.001$), suggesting their inhibition of MX efflux in the MX-selected resistant cells (Fig. 1A; Table I). MX accumulation was increased 5-fold by FTC (10 μ M) ($p < 0.001$) in MCF-7/MX100 cells, but was not altered in MCF-7/sensitive cells, which lack BCRP, based on Western blot analysis (14) (Fig. 1A; Table I). BITC, PEITC, PBITC, PPITC and PHITC produced the greatest effects and were able to increase intracellular MX accumulation by more than 5-fold, higher than that caused by FTC. The seven ITCs showing effects in MCF-7/MX100 cells also increased MX accumulation in MCF-7/sensitive cells ($p < 0.05$ for BITC and $p < 0.001$ for the other ITCs), but to a much smaller extent (Fig. 1A; Table I). AITC, sulforaphane, erucin and erysolin did not alter MX accumulation in MCF-7/MX100 and MCF-7/sensitive cells; PITC did not alter MX accumulation in MCF7/MX100 cells but had a small effect in the sensitive cells ($p < 0.001$) (Fig. 1A; Table I). The ITCs active in MCF-7/MX100 cells also significantly increased MX accumulation in the MX-selected NCI-H460/MX20 cells, although to a smaller extent (2.4-3.1 fold; $p < 0.001$) (Fig. 1B; Table I). In NCI-H460/MX20 cells, FTC (10 μ M) increased the intracellular MX level

by 2.6-fold ($p < 0.001$); in NCI-H460 cells, the BCRP-negative cells, accumulation of MX in the presence or absence of FTC was unchanged (Fig. 1B; Table I). As in MCF-7/MX100 cells, the highest intracellular MX levels in NCI-H460/MX20 cells were observed for those treated by PHITC, PEITC and PBITC, and were 3-fold greater than MX accumulation in cells not treated with ITCs. BITC ($p < 0.001$), PEITC ($p < 0.05$) and PPITC ($p < 0.001$) increased MX accumulation significantly, but slightly, in the NCI-H460 cells. AITC, PITC, sulforaphane, erucin and erysolin had no effect in both NCI-H460/MX20 and NCI-H460 cells (Fig. 1B; Table I). Using DOX as a BCRP substrate, we also found that ITCs reversed its intracellular accumulation in both MCF-7/MX100 cells and NCI-H460/MX20 cells, with a small or no effect in MCF-7/sensitive and NCI-H460 cells (data not shown). A significant correlation was seen for MX accumulation in MCF-7/MX100 cells and that in NCI-H460/MX20 cells ($r^2 = 0.86$; $p < 0.01$; Fig. 1C).

Concentration-dependent Effect of ITCs on the Intracellular Accumulation of MX

To determine whether the effects of ITCs on MX accumulation were concentration dependent, accumulation studies using a series of ITC concentrations (1, 5, 10, 30 and 50 μM) were performed. BITC, PEITC, NITC, HITC, PBITC, PPITC and PHITC, the ITCs that significantly increased MX accumulation at a concentration of 50 μM , were tested in both MCF-7/MX100 and NCI-H460/MX20 cells. With an increase in ITC concentration, the intracellular accumulation of MX generally increased correspondingly, demonstrating dose dependence in both cell lines (Fig. 2). In MCF-7/MX100 cells, all the tested ITCs had significant effects on MX accumulation at 30 μM ($p < 0.001$). In addition, 10 μM of NITC ($p < 0.001$) or HITC ($p < 0.001$), and 5 μM of PHITC ($p < 0.001$) or PBITC ($p < 0.05$) significantly increased intracellular MX accumulation. No significantly greater increase in inhibition was observed at ITC concentrations of 50 μM in MCF-7/MX100 cells for NITC, HITC and PHITC, and IC₅₀ values were around 10 μM (Fig. 2). Similarly, in NCI-

H460/MX20 cells, all the tested ITCs significantly increased MX accumulation at a concentration of 30 μ M ($p < 0.001$), and significant effects were also seen at 10 μ M of PHITC ($p < 0.001$) or PBITC ($p < 0.001$), and 5 μ M of NITC ($p < 0.001$) (Fig. 2). Except PEITC, all the other tested ITCs reached the maximal effect in NCI-H460/MX20 cells at 50 μ M; and IC₅₀ values were around 10 μ M for BITC, PBITC, HITC and PPITC, and 5 μ M for NITC and PHITC (Fig. 2). None of tested ITCs was effective at 1 μ M. For the tested compounds, PHITC, NITC and PBITC were the most potent ITCs in inhibiting the BCRP-mediated efflux of MX.

Effect of ITCs on MX Cytotoxicity

MX cytotoxicity studies were conducted in order to confirm that the increased intracellular MX in BCRP-overexpressing cells produced by ITC exposure also resulted in improved cytotoxicity of MX in the presence of ITCs. NCI-H460/MX20 and NCI-H460 cells were treated by fixed doses of test compounds in combination with increasing concentrations of MX. In NCI-H460/MX20 cells, 10 μ M of the tested ITCs generally inhibited cell growth significantly ($p < 0.001$), with IC₅₀ values ranging from 9.48 to 23.3 nM of MX, lower than that of the cells treated only with MX (41.7 nM) (Table II). FTC, the positive control compound, reduced the IC₅₀ of MX to 0.65 nM in NCI-H460/MX20 cells, whereas there was no significant effect by FTC in the parental cells (Table II). Consistent with the MX accumulation study, the most potent ITCs were PHITC, NITC and PBITC, 10 μ M of which reduced IC₅₀ values of MX in NCI-H460/MX20 cells by 4.4-, 2.5- and 3.8-fold, respectively (Table II). All the tested ITCs, when administered alone, did not inhibit the growth of NCI-H460 cells at the maximal tested concentrations, although NITC induced cell growth in NCI-H460 cells and increased the IC₅₀ of MX by 1.8-fold (Table II). The IC₅₀ values of NCI-H460/MX20 cells were more than 1000 times those of NCI-H460 cells and none of the tested compounds, including FTC, was able to reverse MX cytotoxicity completely back to the level

of sensitive cells (Table II). Using ITC concentrations of 5, 10 and 20 μ M, concentration-dependent effects were examined for NITC and PHITC, two of the most potent ITCs according to the MX accumulation studies, in NCI-H460/MX20 cells. As shown in Fig. 3, the higher the ITC concentrations, the more the cell growth curve shifted to the left, indicating increased sensitivity of the resistant cells to MX cytotoxicity. Both compounds significantly reduced IC₅₀ values of MX to NCI-H460/MX20 cells at 5 μ M ($p < 0.001$), 10 μ M ($p < 0.001$) and 20 μ M ($p < 0.001$), and the values were 20.1, 16.7 and 9.63nM for NITC, respectively, and 14.3, 9.48 and 6.03nM for PHITC, respectively (Table II). These trends suggested that the ITC-mediated effects on MX cytotoxicity were dependent on ITC concentration.

Intracellular PEITC Accumulation in NCI-H460 and NCI-H460/MX20 Cells

To gain insight into the mechanism underlying the effect of ITCs on BCRP, intracellular accumulation of ¹⁴C-PEITC was examined in NCI-H460 and NCI-H460/MX20 cells. Cells were treated by 5 μ M PEITC with or without FTC or GF120918, the two BCRP inhibitors. As shown in Fig. 4, co-incubation of PEITC and 10 μ M FTC or 2.5 μ M GF120918 in NCI-H460/MX20 cells enhanced PEITC accumulation markedly by 7.4- ($p < 0.001$) or 3.7-fold ($p < 0.05$), respectively, indicating that the inhibition of BCRP resulted in more PEITC retained in the resistant cells. In comparison, the intracellular PEITC levels in NCI-H460 cells were unchanged in the presence or absence of either FTC or GF120918 (Fig. 4). In the presence of 5, 10, 25, 50 or 100 μ M of PEITC, the intracellular levels of PEITC in NCI-H460/MX20 cells were all substantially lower than those in the corresponding NCI-H460 cells ($p < 0.001$), being only 11, 8, 17, 26 and 47% of the levels in the sensitive cells, respectively; addition of 10 μ M FTC in NCI-H460/MX20 cells significantly improved PEITC accumulation by 6.8, 4.6, 4.1, 2.7 and 1.5 fold, respectively ($p < 0.001$) (Fig. 5). These results suggest that PEITC and/or its cellular metabolites were indeed transported by BCRP. PEITC accumulation was not altered by the presence of FTC in NCI-H460 cells for

all concentrations except 25 μ M, where there was a small change ($p < 0.05$) (Fig. 5). Based on the fact that PEITC and/or its cellular metabolites are likely to be BCRP substrates, other BCRP substrates may compete with PEITC and thereby affect its intracellular accumulation. Therefore, both NCI-H460/MX20 and NCI-H460 cells were co-incubated with 5 μ M of PEITC and equal molar concentrations of MX or DOX, or higher molar concentrations of MX (10 and 20 μ M). In NCI-H460/MX20 cells, the intracellular level of PEITC remained the same regardless of the presence of MX or DOX (Fig. 6). A similar phenomenon was observed when as high as 20-fold more of MX (100 μ M) than PEITC (5 μ M) was co-incubated with NCI-H460/MX20 cells (data not shown).

DISCUSSION

Like P-gp and MRP1, BCRP transports a variety of chemotherapeutic drugs resulting in MDR, a major reason for treatment failure in cancer therapy. Due to its high expression in normal tissues, BCRP also plays an important role in drug bioavailability, disposition and excretion. In the human jejunum, BCRP transcript is more abundant than MDR1 (for P-gp) with the rank order: BCRP \approx MRP2 > MDR1 (8). The mRNA levels of ABC transporters are reported to correlate with the protein levels and BCRP showed a good correlation between transcript and protein levels in breast cancer (17). Therefore, the impact of BCRP in food-drug interactions may be important.

In this study, we examined the effect of ITCs, which represent a class of dietary components, on BCRP-mediated transport in both resistant and sensitive human breast cancer (MCF-7/MX100 and MCF-7/sensitive) and large cell lung carcinoma (NCI-H460/MX20 and NCI-H460) cells. Significant increases in MX accumulation were observed for BITC, HITC, PEITC, NITC, PHITC, PPITC and PBITC at concentrations of 50 μ M in both MCF-7/MX100 and NCI-H460/MX20 cells, suggesting their inhibitory effect on BCRP. AITC, PITC, sulforaphane, erucin and erysolin did not affect MX accumulation in either of the BCRP-overexpressing cell lines. The increase of intracellular MX accumulation in MCF-7/MX100 cells was remarkable, ranging from 3.5 to 5.8 fold, and comparable to the positive control compound FTC (5 fold). Likewise, a similar trend was observed in NCI-H460/MX20 cells, although to a lower extent (2.4 to 3.1 fold) than in MCF-7/MX100 cells, and the intracellular MX accumulation between MCF-7/MX100 cells and NCI-H460/MX20 cells exhibited good correlation ($r^2 = 0.86$; $p < 0.01$). These findings suggest that the increased accumulation of MX caused by ITCs is BCRP related, and indicate that ITCs may be BCRP inhibitors. All the seven ITCs that increased MX accumulation in resistant cells produced small but significant effects in MCF-7/sensitive cells, and BITC, PEITC and PPITC enhanced

MX accumulation in NCI-H460 cells slightly but significantly as well. PITC, which did not show any effect in the resistant cells, enhanced MX accumulation in MCF-7/sensitive cells significantly. The underlying mechanisms for the increased MX accumulation in the parental cells are not known. Western blot analysis showed that neither of the parental cell lines had detectable BCRP, P-gp or MRP1 expression, although this does not preclude the existence of low levels of these transporters in the cells (14). In fact, low expression of BCRP mRNA in NCI-H460 cells, as detected by Northern blot analysis, has been reported (18). However, FTC, a BCRP inhibitor with high potency, did not show any effect in the parental cells. Our laboratory previously investigated organic ITCs as inhibitors of P-gp- and MRP1-mediated drug resistance in cancer cell lines and demonstrated that some ITCs can inhibit both transporters at concentrations of 50 μ M, but had no effects at 10 μ M (13). Therefore, ITCs only represent weak inhibitors of P-gp or MRP1; additionally, MX has been shown to be a weak P-gp substrate (19,20) but not a substrate for MRP1 (21). Therefore, it is possible that low expression of BCRP and/or P-gp in the parental cell lines may be responsible for the increased MX accumulation, or that there may be another transporter or mechanism involved in the ITC-induced increases in MX accumulation in the parental cell lines.

Concentration-dependent effects of the ITCs on MX accumulation were observed. The ITCs were effective at concentrations of 5 μ M (NITC, PHITC and PBITC), 10 μ M (HITC) or 30 μ M (BITC, PEITC and PPITC). In both MCF-7/MX100 and NCI-H460/MX20 cells, PHITC, NITC and PBITC appeared to be the most potent ITCs in reversing MX accumulation among the tested compounds. None of the ITCs exhibited significant effects at 1 μ M. The results from MX cytotoxicity studies confirmed the MX accumulation study findings, in that ITCs with the most potent effects on MX accumulation had the most potent effects on the cytotoxicity of MX. On the whole, IC₅₀ values of MX cytotoxicity were reduced significantly by 10 μ M of ITCs in NCI-H460/MX20 cells. The ITCs did not increase

MX cytotoxicity in the sensitive NCI-H460 cells at the maximum concentration used in the cytotoxicity studies, but 20 μ M of NITC reduced MX cytotoxicity as represented by a higher IC₅₀ value than control. Concentration-dependent effects on MX cytotoxicity were also observed. Taken together, the results of both accumulation and cytotoxicity studies suggested that ITCs are BCRP inhibitors. It appears that inhibitory activity is related to lipophilicity of the compounds, because the three most potent compounds, PHITC, PBITC and NITC, are the most lipophilic, whereas those inactive (erucin, eysolin, sulforaphane, AITC and PITC) are the most hydrophilic compounds among the tested ITCs. All of the effective ITCs contained a phenyl ring except for HITC, but HITC has high lipophilicity. PITC, with a phenyl ring but a short backbone, did not inhibit BCRP at the tested concentration, suggesting both the backbone length and phenyl ring of ITCs may play a role in BCRP inhibition.

The concentration of ITCs that inhibit BCRP may be clinically relevant. It is estimated that milligrams of ITCs are consumed by humans per day through dietary vegetable sources (12). Some estimates of human consumption of glucosinolates, the ITC precursors, are as high as 300 mg/d (~660 μ mol/d) (11). Recently, a number of food supplements containing ITCs or extracts of cruciferous vegetables have become commercially available. If 1.65 L is taken as the estimated gut volume (22) and 10 mg as the PEITC (~60 μ mol) intake from the diet, the concentration of PEITC would be 37 μ M. In a Phase I clinical study evaluating PEITC as a chemopreventive agent, a single oral dose of 40 mg was administered to subjects (23). Using 1.65 L as the intestinal fluid volume, this amount will produce an intestinal concentration of 148 μ M of PEITC. On the other hand, luminal contents are mixed predominantly within smaller segments of the small intestine (24), suggesting local or segmental concentrations of a ingested food component in the intestine may be higher. Moreover, intracellular concentrations of ITCs are much higher than extracellular concentrations. For example, cells exposed to 100 μ M concentrations of sulforaphane have

intracellular concentrations of 6.4 mM (parent and GSH conjugate) (25). The minimal effective concentration of ITCs for BCRP inhibition ranged from 5 to 30 μ M according to our study. The bioavailability of PEITC is high and clearance is low in rats (Ji Y and Morris ME, unpublished data), so little presystemic metabolism would be expected, in contrast to many other dietary components. As a result, ITC concentrations in the intestine may be high enough to affect BCRP-mediated transport.

PEITC is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low *in vivo* toxicity. Using PEITC as a model drug, we examined the intracellular accumulation of 14 C-PEITC in BCRP-overexpressing (NCI-H460/MX20) and BCRP-negative (NCI-H460) cells, in order to elucidate whether PEITC is a BCRP substrate. When the cells were incubated with different concentrations of PEITC ranging from 5 to 100 μ M, the intracellular levels of PEITC in NCI-H460/MX20 cells were only 8-47% of those in the corresponding parental cells ($p < 0.001$). Co-administration of 10 μ M FTC or 2.5 μ M GF120918 enhanced 14 C-PEITC accumulation in NCI-H460/MX20 cells by 7.4 fold ($p < 0.001$) or 3.7 fold ($p < 0.05$), respectively; FTC or GF120918 did not alter PEITC accumulation in the parental NCI-H460 cells. Overall, these results suggest that PEITC and/or its cellular metabolites are BCRP substrates. Increasing the PEITC concentration resulted in a decrease in the percentage of intracellular PEITC concentration in NCI-H460/MX20 cells compared with that in NCI-H460 cells; the effect of FTC in increasing the accumulation of 14 C-PEITC was also decreased in NCI-H460/MX20 cells as PEITC concentration increased. Therefore, the BCRP-mediated transport of PEITC and/or metabolites appears to be saturable, supporting our hypothesis that PEITC is actively transported by BCRP. ITCs enter cells by passive diffusion and primarily form ITC-glutathione (-GSH) conjugates in cells. The intracellular accumulation of ITC-GSH is maximal after about 30 min in cultured cells (26). It was reported recently that AITC, BITC,

and PEITC, were rapidly exported, mainly in the forms of GSH- and cysteinylglycine-conjugates, by P-gp and MRP1 (27). MRPs transport GSH-, glucuronide-, or sulfate-conjugates or cotransport substrates with GSH (28). BCRP is known to transport sulfate conjugates (5), but it may not be important in the transport of glucuronide conjugates (29). No evidence is available concerning the transport of GSH conjugates by BCRP. Hence, whether PEITC or PEITC-GSH represents BCRP substrates is not known at this time.

In our studies, two known BCRP substrates, MX and DOX, in equal or higher molar concentrations, did not affect PEITC intracellular accumulation in BCRP-overexpressing cells, implying that PEITC and/or its cellular metabolites interact with BCRP through a different mechanism. It is possible that BCRP has more than one binding site with distinct specificity for different substrates. Furthermore, as a half-transporter, BCRP may form a multiprotein complex to perform its function. Substrate interaction studies using BCRP expressed in oocytes of *Xenopus laevis* reported that no two substrates reciprocally inhibited the efflux of the other, suggesting the complexity of substrate binding with BCRP homodimers (30). For example, flavopiridol could inhibit MX transport, but the reverse was not true (30). Another example is indolocarbazole compound A, a chemotherapeutic agent, which is a BCRP substrate but its transport is not inhibited by MX (31).

In conclusion, we have shown that organic ITCs reversed intracellular MX accumulation in BCRP-overexpressing MCF-7/MX100 and NCI-H460/MX20 cells, with small or no effect in BCRP-negative MCF-7/sensitive and NCI-H460 cells. ITCs exerted activity on intracellular MX accumulation in the resistant cells in a concentration-dependent manner, and significant effects were observed at ITC concentrations of 5, 10 or 30 μ M. ITCs generally potentiated MX cytotoxicity to BCRP-overexpressing cells at concentrations of 5 or 10 μ M but had no or small effects in BCRP-negative cells. Among the tested ITCs, PHITC, NITC and PBITC possessed the highest potencies. In addition, PEITC and/or its cellular

metabolites may represent BCRP substrates. Our results demonstrate that organic ITCs inhibit BCRP-mediated transport and suggest the potential clinical significance of intestinal ITC-drug interactions.

ACKNOWLEDGEMENTS

We are grateful to Dr. Susan E. Bates (National Cancer Institute) for providing MCF-7/MX100, MCF-7/sensitive, NCI-H460/MX20 and NCI-H460 cells, and FTC. We thank Qi Wang for synthesizing and characterizing ¹⁴C-PEITC. Financial support for this work was provided by U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376.

ABBREVIATIONS: ABC, ATP-binding cassette; AITC, allyl isothiocyanate; BCRP, breast cancer resistance protein; BITC, benzyl isothiocyanate; FTC, fumitremorgin C; GSH, glutathione; HITC, hexyl isothiocyanate; ITC, isothiocyanate; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; MX, mitoxantrone; NITC, 1-naphthyl isothiocyanate; PBITC, phenylbutyl isothiocyanate; PEITC, phenethyl isothiocyanate; P-gp, P-glycoprotein; PHITC, phenylhexyl isothiocyanate; PITC, phenyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; SRB, sulforhodamine B.

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TABLES

Table I. Effect of ITCs on MX accumulation in the parental and MX-selected MCF-7 and NCI-H460 Cells

| Compounds | Cell Lines | | | |
|--------------|-----------------|--------------|---------------|---------------|
| | MCF-7/sensitive | MCF-7/MX100 | NCI-H460 | NCI-H460/MX20 |
| control | 100 ± 7.36 | 100 ± 5.73 | 100 ± 11.9 | 100 ± 10.7 |
| AITC | 100 ± 13.6 | 120 ± 27.8 | 98.1 ± 8.78 | 104 ± 7.93 |
| BITC | 131.5 ± 38.8* | 506 ± 110** | 162 ± 49.6** | 296 ± 38.3** |
| NITC | 135 ± 21.7** | 409 ± 79.9** | 112 ± 27.2 | 257 ± 47.2** |
| PEITC | 160 ± 15.2** | 564 ± 171** | 151.4 ± 35.3* | 315 ± 87.8** |
| PITC | 147 ± 29.4** | 141 ± 20.2 | 108.8 ± 28.6 | 111 ± 11.9 |
| sulforaphane | 102 ± 3.1 | 134 ± 19.7 | 102 ± 6.89 | 137 ± 20.9 |
| erucin | 98.8 ± 9.9 | 164 ± 20.3 | 99.1 ± 11.0 | 159 ± 46.5 |
| erysolin | 105.5 ± 6.9 | 169 ± 28.4 | 116 ± 26.9 | 131 ± 19.2 |
| PBITC | 174 ± 16.9** | 582 ± 72.4** | 140 ± 45.7 | 306 ± 66.5** |
| PPITC | 143 ± 36.2** | 532 ± 81.1** | 169 ± 34.1** | 260 ± 111** |
| HITC | 134 ± 23.1** | 353 ± 60.9** | 109 ± 39.7 | 243 ± 95.2** |
| PHITC | 192 ± 18.6** | 518 ± 120** | 137 ± 67.8 | 398 ± 103** |
| FTC | 110 ± 8.9 | 499 ± 111** | 126 ± 16.0 | 255 ± 45.8** |

Cells were incubated with 3 μM MX at 37°C in the absence or presence of ITCs (50 μM) for 30 min and MX accumulation was measured by flow cytometric analysis. FTC (10 μM) was used as a positive control. The accumulation of MX was expressed as percent of control, where control represents the MX accumulation in the absence of ITCs. The data are expressed as mean ± SD, n = 9. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's post hoc test; *p < 0.05; **p < 0.001.

Table II. Effect of ITCs on MX cytotoxicity in parental and MX-selected NCI-H460 Cells

| | IC ₅₀ (pM) (NCI-H460) | | IC ₅₀ (nM) (NCI-H460/MX20) | | |
|---------------------------|-------------------------------------|----------------|--|----------------|----------------|
| | Control | 4.23 ± 1.78 | 41.7 ± 12.4 | | |
| ITC concentration (μM) | 10 | 20 | 5 | 10 | 20 |
| BITC | 2.95 ± 0.75 | | | 19.8 ± 3.93 ** | |
| PEITC | 2.21 ± 0.66 | | | 23.3 ± 9.54 ** | |
| NITC | | 7.96 ± 2.02 ** | 20.1 ± 0.86 ** | 16.7 ± 4.59 ** | 9.63 ± 3.67 ** |
| PBITC | 4.31 ± 1.12 | | | 11.0 ± 3.84 ** | |
| PPITC | 3.83 ± 1.49 | | | 17.1 ± 8.99 ** | |
| HITC | | 2.08 ± 0.71 | | | 10.9 ± 1.73 ** |
| PHITC | | 2.80 ± 1.59 | 14.3 ± 2.73 ** | 9.48 ± 2.70 ** | 6.03 ± 1.35 ** |
| FTC | 2.13 ± 0.72 | | | 0.65 ± 0.31 ** | |

Cells were grown in 96-well plates for 2 days, and incubated with MX of increasing concentrations of 0-300 or 0-1000 nM (for NCI-H460 or NCI-H460/MX20 cells, respectively) at 37°C in the absence or presence of specified concentrations of ITCs for 1 day, followed by incubation in fresh media for another day. FTC (10 μM) was used as a positive control. Cell growth was determined using a SRB assay. IC₅₀ values were determined by fitting the cell growth curve as described in Materials and Methods. The data are expressed as mean ± SD, n = 1 experiment performed in quadruplicate for NCI-H460 cells or n = 3 or 4 experiments performed in quadruplicate for NCI-H460/MX20 cells. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's post hoc test; **p < 0.001.

Figure-caption List

Fig. 1. Effect of ITCs on the intracellular accumulation of MX in the parental and MX-selected MCF-7 and NCI-H460 cells. The 30-min accumulation of 3 μ M MX was measured in the absence (0.1% DMSO) or presence of various ITCs (50 μ M) in MCF-7/sensitive and MCF-7/MX100 cells (A) as well as in NCI-H460 and NCI-H460 /MX20 cells (B). The intracellular accumulation of MX is expressed as percent of control, where control represents the MX accumulation in the absence of ITCs. Data are expressed as mean \pm SD, n = 9. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's post hoc test; *p < 0.05; **p < 0.001. (C) Relationship between MX accumulation in MCF-7/MX100 and in NCI-H460/MX20 cells. Each point represents the mean value of the percent of control (n = 9) and the line was generated from linear regression with an r^2 of 0.86 (p < 0.01).

Fig. 2. Concentration-dependent effect of ITCs on MX accumulation in MCF-7/MX100 and NCI-H460/MX20 cells. Cells were incubated with 1, 5, 10, 30 and 50 μ M of BITC (A), PEITC (B), NITC (C), HITC (D), PBITC (E), PPITC (F), or PHITC (G). The intracellular accumulation of MX was determined by flow cytometric analysis and expressed as percent of control (in the absence of ITCs) in MCF-7/MX100 cells (●, solid line) or NCI-H460/MX20 cells (○, dashed line). The data are expressed as mean \pm SD, n = 9. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's post hoc test; *p < 0.05 and **p < 0.001 compared to the corresponding controls.

Fig. 3. The concentration-dependent effect of NITC and PHITC on the MX cytotoxicity in NCI-H460/MX20 cells. Cells were grown in 96-well plates for 2 days, and incubated with increasing concentrations of 0-1000 nM MX at 37°C in the absence (●) or presence of 5 (○), 10 (▲) and 20 (△) μ M of NITC (A) or PHITC (B) for 1 day, followed by incubation in fresh

media for another day. Cell growth was determined by a SRB assay and expressed as cell survival fraction, which was calculated as described in Materials and Methods. The data are expressed as mean \pm SD; n = 3 or 4 experiments, each performed in quadruplicate.

Fig. 4. PEITC accumulation in the parental NCI-H460 and MX-selected NCI-H460/MX20 cells. Cells were incubated with 5 μ M 14 C-PEITC in the absence or presence of FTC (10 μ M) or GF120918 (2.5 μ M) in sodium buffer for 1 hour. PEITC accumulation was normalized for cellular protein and presented as percent of control, where control represents cells treated with PEITC but without any modulator. Data are expressed as mean \pm SD, n = 6; *p < 0.05 and **p < 0.001 by a one-way ANOVA followed by Dunnett's post-hoc test.

Fig. 5. Concentration-dependent PEITC accumulation in the parental NCI-H460 and MX-selected NCI-H460/MX20 cells. Cells were incubated with specified concentrations of 14 C-PEITC (supplemented with cold PEITC) in the absence or presence of FTC (10 μ M) for 1 hour. The intracellular PEITC accumulation was normalized by the amount of cellular protein. Data are expressed as mean \pm SD, n = 6. Statistical analysis was conducted by a student's t-test; ^a significantly different compared to NCI-H460 cells treated with the same concentration of PEITC in the absence of FTC and p < 0.001 (p < 0.05 for the NCI-H460 cells treated with 25 μ M PEITC in the presence of 10 μ M FTC); ^b significantly different compared to NCI-H460/MX20 cells treated with the same concentration of PEITC in the absence of FTC and p < 0.001.

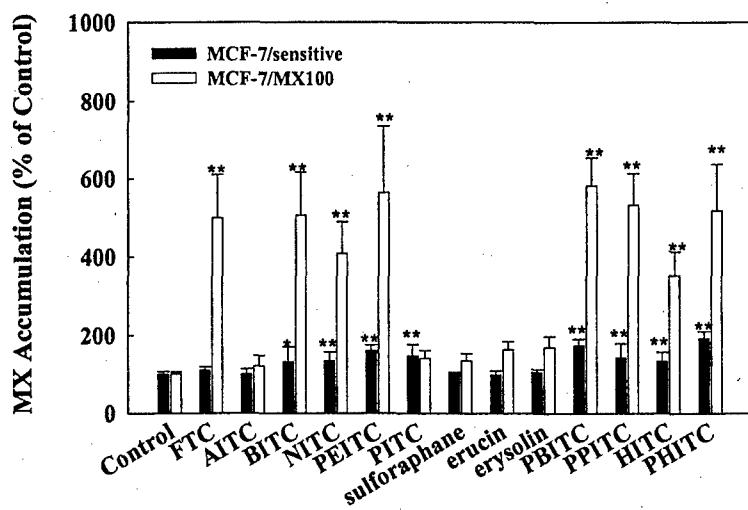
Fig. 6. Effects of MX and DOX on PEITC accumulation in the parental NCI-H460 and MX-selected NCI-H460/MX20 cells. Cells were incubated with 5 μ M 14 C-PEITC in the absence or presence of DOX (5 μ M) or MX (5, 10, 20 μ M) for 1 hour. The intracellular PEITC

accumulation was normalized by the amount of cellular protein and presented as percent of control, where control represents cells treated with PEITC alone. Data are expressed as mean \pm SD, n = 6.

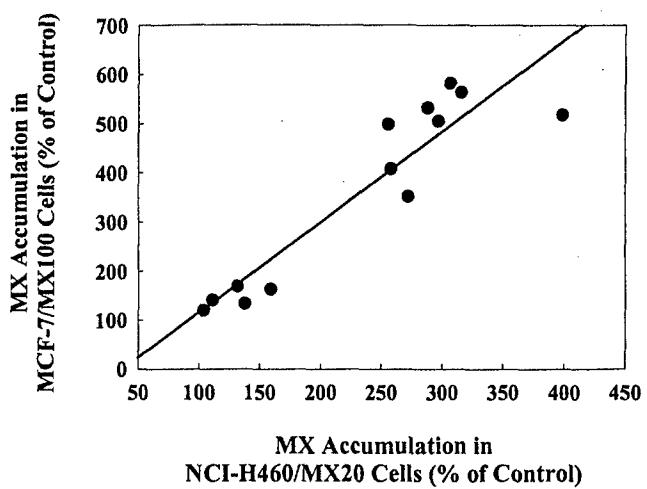
FIGURES

Fig. 1.

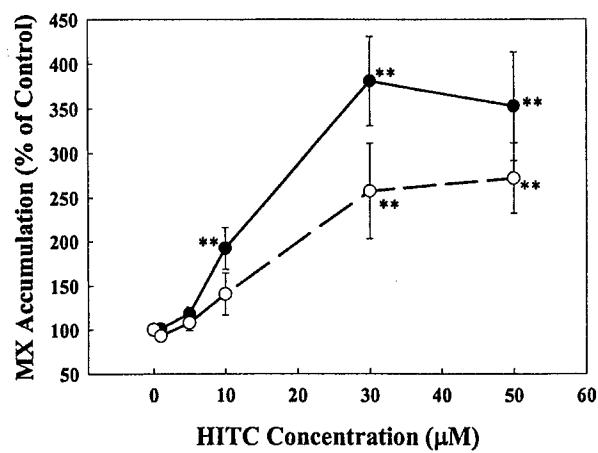
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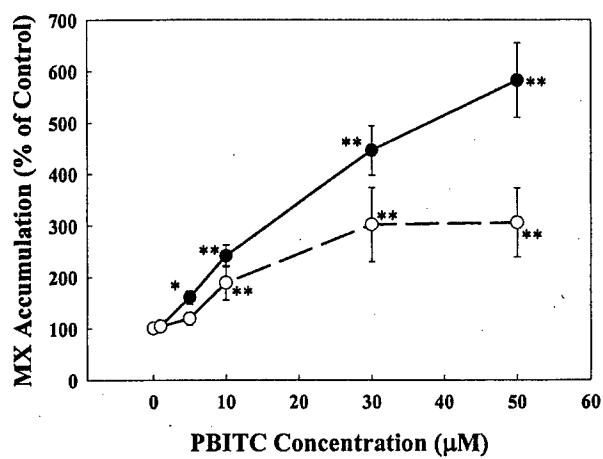
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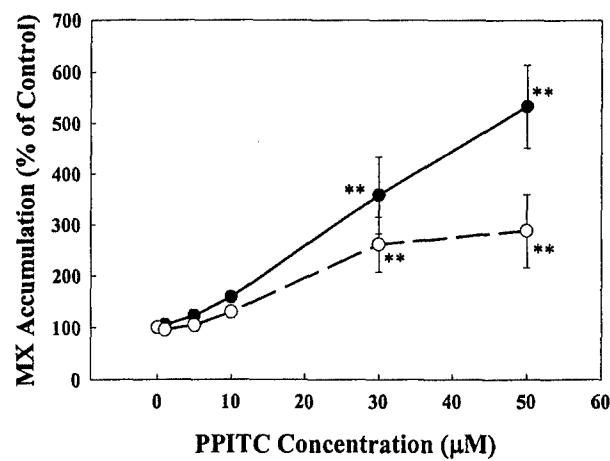
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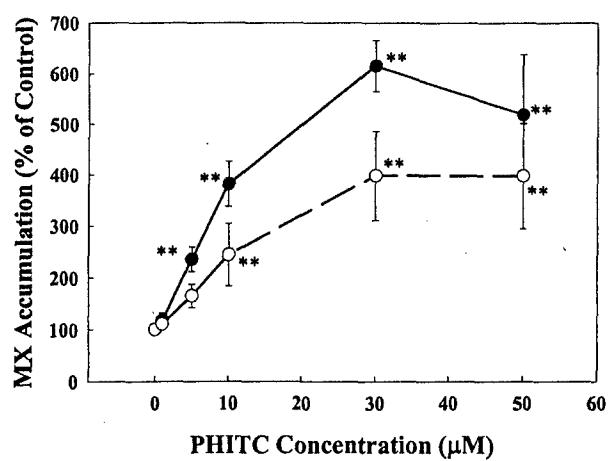
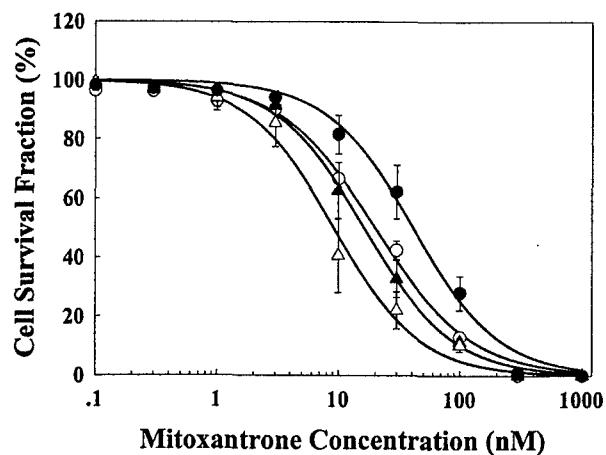


Fig. 3.

A



B

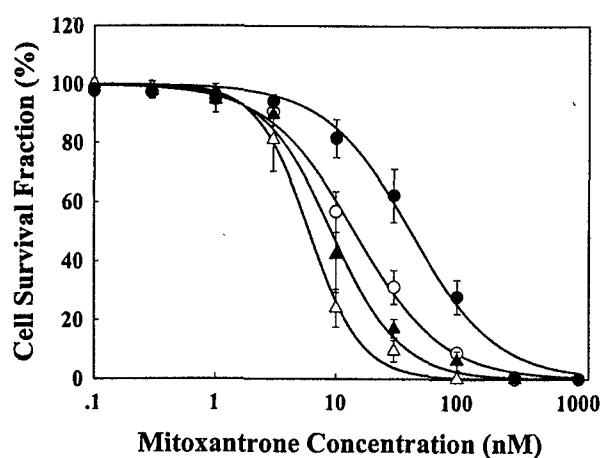


Fig. 4.

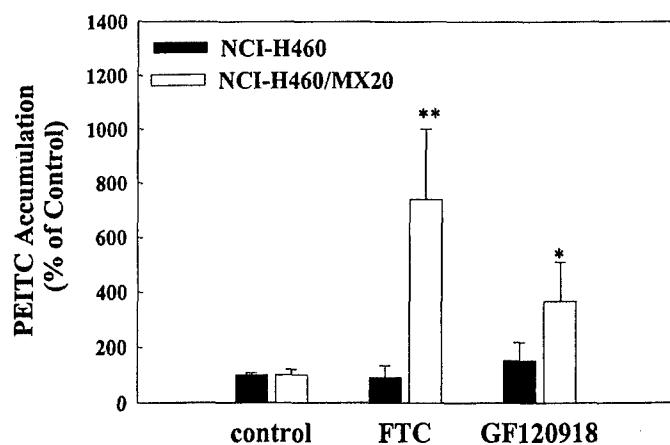


Fig. 5.

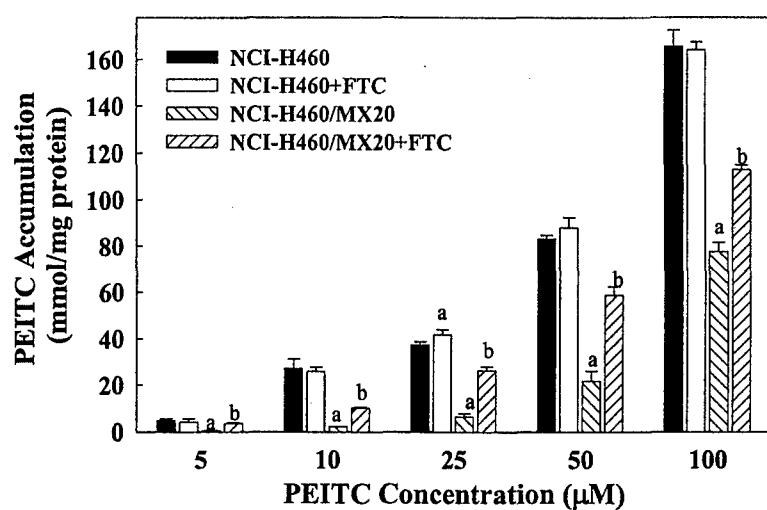
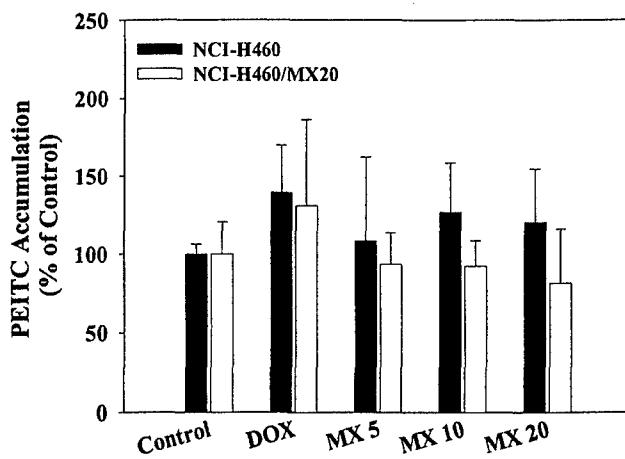


Fig. 6.



Pharmacokinetics of α -Naphthyl Isothiocyanate in Rats

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c) Document statistics:

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number of words in the Results...

number of words in the Conclusion...

d) Abbreviations: ABC, ATP binding cassette; AIC, Akaike's Information Criterion; BITC,

benzl isothiocyanate; BSO, buthionine sulfoximide; CYP, cytochrome P450; DNM,

daunomycin; EHC, enterohepatic cycling; GI, gastrointestinal; GSH, glutathione; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; ITCs, isothiocyanates; iv, intravenous; LLOQ, lower limit of quantitation; MDR, multidrug resistance; MRP, multidrug resistance associated protein; MW, molecular weight; 1-NA, α -naphthyl amine; NE, naphthalene; 1-NIC, α -naphthyl isocyanate; 1-NITC, α -naphthyl isothiocyanate; PEITC, phenethyl isothiocyanate; P-gp, P-glycoprotein; po, oral; QC, quality control; SC, Schwarz Criterion; VBL, vinblastine

- e) Recommended sections: Absorption, Distribution, Metabolism and Excretion

Abstract

Many naturally occurring and synthetic isothiocyanates (ITCs) showed activities to inhibit chemical carcinogenesis in animal models. More recently, we found that α -naphthyl isothiocyanate (1-NITC), a synthetic ITC, significantly increased the accumulation of the anticancer drug daunomycin in P-glycoprotein (P-gp) and multidrug resistance associated protein 1 (MRP1) overexpressed cells, indicating the potential application of 1-NITC as a chemosensitizing agent for cancer chemotherapy [Hu and Morris, *J. Pharm. Sci.* in press]. The objective of this study was to explore the pharmacokinetic characteristics of 1-NITC in rats. A single dose of 10, 25, 50 or 75 mg/kg of 1-NITC was administered intravenously or orally to female Sprague-Dawley rats ($n = 4$ for each group). Dose-normalized concentration-time profiles were not superimposable following intravenous or oral dosing groups, indicating that the disposition of 1-NITC in rats was nonlinear. As doses increased from 10 to 75 mg/kg following intravenous administration, the total clearance (CL) decreased from 2.2 ± 0.9 to 0.8 ± 0.3 L/h; oral availability averaged 0.46 for oral doses of 10-75 mg/kg. A nonlinear two-compartment open model with capacity-limited absorption and capacity-limited elimination from the central compartment best fit the data, based on goodness-of-fit criteria. The mechanism underlying the nonlinear elimination of 1-NITC in rats is most likely due to the capacity-limited metabolism of 1-NITC mediated by cytochrome P450 and glutathione S-transferase enzymes. We propose that the nonlinear absorption of 1-NITC is not due to saturable transport in the intestine, but reflects a rate-limiting step, the active transport of the labile glutathione conjugate of 1-NITC into bile, followed by its rapid hydrolysis and the rapid absorption of the lipophilic 1-NITC. This study represents the first report of the pharmacokinetics of 1-NITC; this information will be useful for optimizing dosing regimens of 1-NITC, as a multidrug resistance reversal agent.

Keywords: α -naphthyl isothiocyanate; pharmacokinetics; hepatic clearance; biliary excretion; enterohepatic cycling

1. Introduction

Many naturally occurring and synthetic isothiocyanates (ITCs; R-N=C=S) inhibited chemical carcinogenesis in experimental animals (e.g. rats, mice and rabbits) and are being considered as chemopreventive agents for human use¹. As early as the 1960's, α -naphthyl isothiocyanate (1-NITC) (Fig. 1), a synthetic ITC, was reported as a carcinogenesis inhibitor in experimental animal models (rat livers and bladders) induced by 2-acetylaminofluorene, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, 4-dimethylaminozobenzene, 3-methyl-4-dimethylaminozo benzene, ethionine, *N*-nitrosodiethylamine, and *m*-toluylenediamine^{2 3 4 5 6}. Extensive studies have shown that benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), two naturally occurring ITCs present in cruciferous vegetables (e.g. watercress, broccoli, cabbage and cauliflower), protect against cancer by inhibition of phase I cytochrome P450 (CYP) enzymes responsible for carcinogen activation⁷, induction of phase II enzymes such as glutathione *S*-transferase (GST) responsible for the detoxification of electrophilic intermediates^{7 8}, and induction of apoptosis of cancer cells⁹.

Recently, we have found that 1-NITC significantly increased the accumulation of the anticancer drugs daunomycin (DNM) and vinblastine (VBL) in P-glycoprotein (P-gp) and multidrug resistance associated protein 1 (MRP1) overexpressed human cancer cells¹⁰ [Hu and Morris, *J. Pharm. Sci.* in press]. In our previous studies, 1-NITC significantly increased the 2-h accumulation of DNM in human breast cancer MCF-7/ADR cells (P-gp overexpression) and pancreatic adenocarcinoma PANC-1 cells (MRP1 overexpression) by 4.9 ± 0.3 and 2.6 ± 0.1 fold, and the accumulation of VBL in both cell lines by 41.8 ± 3.2 and 5.4 ± 0.5 fold greater than

the control groups ¹⁰ [Hu and Morris, *J. Pharm. Sci.* in press]. The activities of 1-NITC were greater than BITC and PEITC by approximately 2-fold.

The disposition of 1-NITC has been predominantly studied using radiolabeled 1-NITC, so that total 1-NITC (parent and radiolabeled metabolites) was quantitated. Capizzo and Roberts found that approximately 70% of the radioactivity after administering ¹⁴C-1-NITC was absorbed from the gastrointestinal (GI) track within 24 h and widely distributed in all body tissues following oral doses of 50, 100 or 300 mg/kg of the compound to rats ¹¹. The liver, kidney, adipose tissues and blood contained accumulated ¹⁴C to a greater extent than spleen, brain, pancreas, heart, lungs, gonads and adrenals ¹¹. The radioactivity was found in all excretion products examined, i.e. about 77% of the administered dose was recovered within 72 h from the urine (38.4%), expired gases (25.8%), and feces (12.4%) ¹¹. Lock et al. performed disposition studies with dually labeled 1-NITC (¹⁴C-isothiocyanate and 4-³H-naphthyl) with a ratio of ³H/¹⁴C of 6.3, following an oral dose of 300 mg/kg to rats ¹². Rats excreted more ³H than ¹⁴C (i.e. ³H/¹⁴C ratio was greater than 6.3) in bile during the first 8 h after 1-NITC administration ¹². Breschi et al. reported that unchanged 1-NITC was present in liver, kidney and blood, but not in brain, urine and bile, following the oral administration of 600 mg/kg 1-NITC to rats, by UV spectrophotometry, thin-layer chromatography and gas chromatography for detection ¹³. The phase I metabolites of 1-NITC, α-naphthyl amine (1-NA) and α-naphthyl isocyanate (1-NIC) (Fig. 1), were identified in rat bile ^{14 15}. GST has been shown to be involved in the Phase II reactions of 1-NITC with the reduced glutathione (GSH) ^{16 17 18 19 20 21 22} and the glutathione conjugate GS-1-NITC (Fig. 1) was identified in rat hepatocytes ¹⁶ and undergoes biliary excretion ^{20 21}.

We have developed a high-performance liquid chromatography (HPLC) assay to determine the concentrations of 1-NITC and its metabolites 1-NA and 1-NIC in rat plasma and urine ²³. The objective of this investigation is to characterize the pharmacokinetics of 1-NITC in rats following intravenous and oral administration. The information will be valuable in further studies to assess the chemotherapeutic properties of 1-NITC in animal studies.

2. Experimental

2.1. Chemicals and reagents

1-NITC was purchased from Sigma (St. Louis, MO) at more than 99% purity. The internal standard naphthalene (NE) was purchased from Fisher Scientific (Fair Lawn, NJ) at more than 99% purity. Acetonitrile was HPLC grade from Fisher.

2.2. Animals

Age-matched (12-15 weeks old) adult female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighting 200-250 g were used throughout the study. The rats underwent acclimatization at least five days before surgery. The rats received regular laboratory chow (Harlan) and allowed free access to water. The rats were randomly into groups and kept in individual cage. The animal housing room had controlled environmental conditions with temperature and relative humidity of approximately $20 \pm 2^{\circ}\text{C}$ and 40-70%, respectively, and artificial lighting, alternating on a 12-h light-dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee at University at Buffalo, State University of New York.

2.3. Doses and sample collection

The cannulas were inserted into rat right jugular veins following an intramuscular injection of ketamine 90 mg/kg and xylazine 10 mg/kg (Henry Schein, Melville, NY). Two or three days following surgery, a single dose of 10, 25, 50 or 75 mg/kg 1-NITC (10 mg/ml) in a vehicle consisting of 5% ethanol (Pharmaco Products, Brookfield, CT), 5% cremophor EL (Sigma), and 90% sterile saline solution (Braun Medical, Irvine, CA) was administered via intravenous bolus through the cannulas or by oral gavages. Four rats were randomly assigned to each group.

Serial blood samples (200 µl each) were collected from the jugular veins at 5, 10, 20, 30 min, 1, 2, 3 (for oral doses), 4, 6, 9, 12, 24, 36 (for 50 and 75 mg/kg doses) and 48 h (for 50 and 75 mg/kg doses) following administration of 1-NITC, and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma was immediately separated from blood via centrifugation at 1,000 × g (Eppendorf Centrifuge 5417R, Brinkmann Instruments Inc., Westbury, NY) for 5 min at 4°C and stored at -80°C until analysis by HPLC assay.

2.4. HPLC Assay

1-NITC plasma concentration levels were measured by a validated reverse-phase HPLC method with UV detection²³.

2.4.1. HPLC instrumentation

The Waters HPLC system (Milford, MA) consisted of a model 1525 binary pump, a model 717plus autosampler (a 250-µl injector and a 200-µl loop) configured with a heater/cooler, a model 5HC column oven, and a model 2487 UV detector. The column and autosampler temperatures were kept at room temperature ($21 \pm 1^\circ\text{C}$) and 4°C, respectively. The reversed-phase chromatography was performed with a Partisphere C-18 5 µm column 125 × 4.6 mm i.d. (Whatman, Clifton, NJ) protected by a RP guard cartridge system C-18 5 µm (Whatman), and eluted isocratically with a mobile phase consisting of acetonitrile-H₂O (70:30, v/v). The flow rate was 1.0 ml/ml and the injection volume was 50 µl. The UV detector was set at a single wavelength of 305 nm. The Breeze System software version 3.2 (Waters) was used for instrument control and data analysis.

2.4.2. Plasma sample preparation

The stock solutions 10 mg/ml of 1-NITC and the internal standard naphthalene (NE) were freshly prepared by dissolving a weighted amount of each compound in acetonitrile. The 0.5 mg/ml working solution of NE was prepared by diluting the stock solution with acetonitrile. Each blank plasma sample (30 µl) was spiked with 3 µl of NE solution, 3 µl of varying concentrations of 1-NITC, and 114 µl acetonitrile, to prepare a series of standards (10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml as final concentration) for the calibration curve. Each plasma sample (30 µl) of pharmacokinetic studies was spiked with 3 µl NE solution and 117 µl acetonitrile, vortexed and centrifuged at 10,000 × g (Eppendorf) for 5 min at 4°C. The resulting supernatants were analyzed for 1-NITC. The quality control (QC) samples at low (10 ng/ml), medium (500 ng/ml) and high concentrations (5,000 ng/ml) were prepared by the same procedures.

2.4.3. Assay validation

The lower limit of quantification (LLOQ) of 1-NITC was determined as 10 ng/ml in plasma. The linearity of the calibration curve was evaluated over a concentration range 10 to 5,000 ng/ml, based on regression analysis of peak area ratios (1-NITC/NE) to 1-NITC concentrations in blank plasma. The accuracy was determined as 95-106% and 97-102%, precision as 98-100% and 93-97%, and absolute recovery as 93-97% and 96-97%, for within-day and between-day analysis, respectively²³.

2.5. Pharmacokinetic analysis

1-NITC plasma concentration versus time data were analyzed by noncompartmental methods, performed by WinNonLin (v 2.1) (Pharsight, Mountain View, CA). The peak plasma concentration (C_{max}) and the time to reach peak concentration (T_{max}) were determined directly from experimental observations. The area under the plasma concentration-time curves from time zero to infinity (AUC_{∞}) was calculated by the log-linear trapezoidal method. The slope (k) of the terminal phase of concentration-time profile was determined by the log-linear regression of at least three data points. The absolute value of k was used to estimate the terminal half-life ($t_{1/2}$) by $t_{1/2} = \ln 2/k$. Following intravenous dosing, total clearance (CL) was estimated by dividing the administered dose by AUC_{∞} . The absolute oral bioavailability of 1-NITC was estimated from the ratios of dose-normalized AUC_{∞} values following oral administration over that obtained following intravenous administration.

Further pharmacokinetic analysis was conducted by simultaneous fitting all intravenous and oral plasma concentration data using ADAPT II software (Biomedical Simulations Resource, Los Angeles, CA) compiled by Digital Visual Fortran 6.0 (Digital Equipment Corp.). The initial estimates for ADAPT II modeling were derived from those obtained from the noncompartmental analysis (WinNonLin 2.1). Primary parameters obtained from ADAPT II analysis for Figs. 6-9 included first-order absorption rate constant (k_a), first-order elimination rate constant from the absorption site (k), first-order distribution rate constants between the central and peripheral compartments (k_{12} and k_{21}), volume of distribution in central compartment (V_c), total clearance (CL), Michaelis-Menten constant (K_m and K'_m), and maximum velocity (V_{max} and V'_{max}). A simple variance model was used as defined by the equation of $\text{var}(C) = (\sigma_1 + \sigma_2 \times Y)^2$, where σ_1 and σ_2 are the variance model parameters, and Y is the model predicted output²⁴. Standard

goodness-of-fit criteria were used for model discrimination, including model convergence, Akaike's Information Criterion (AIC), Schwarz Criterion (SC), estimator criterion value for the maximum likelihood, correlation coefficient (R^2), examination of residuals, and visual inspection. The secondary parameters, volume of peripheral compartment (V_T) and distribution clearance (CL_D), were calculated from the primary parameters by the following equations, i.e.

$$CL_D = k_{12} \times V_c \text{ and } V_T = k_{12} \times V_c / k_{21}.$$

3. Results

The dose-dependent plasma concentration-time profiles following the intravenous administration of 1-NITC to rats are shown in Fig. 2. 1-NITC could be detected in all samples over 48 h in the high-dose groups (50 and 75 mg/kg). However, by 24 h after administration, 1-NITC concentrations were below LLOQ in the low-dose groups (10 and 25 mg/kg). 1-NITC exhibited biexponential disposition following intravenous administration, but the elimination of 1-NITC from plasma was nonlinear. Convex plasma concentration-time profiles were observed following the highest dose administration. The dose-normalized plasma concentration-time profiles, presented in Fig. 3, were not superimposable.

The dose-dependent plasma concentration-time profiles of 1-NITC following oral administration to rats are shown in Fig. 4. Following the administration of 10 and 25 mg/kg, 1-NITC concentrations could be determined over 24 h. 1-NITC could be detected in plasma samples over 36 and 48 h, respectively, following the 50 and 75 mg/kg doses. The average time to maximum concentration (T_{max}) was determined as 0.5, 1, 4 and 6 h for 10, 25, 50 and 75 mg/kg dosing groups, respectively. The dose normalized plasma concentration-time profiles, plotted in Fig. 5, were not superimposable, again suggesting nonlinearity of 1-NITC kinetics in rats.

Noncompartmental analysis was performed by WinNonLin (v. 2.1) software to obtain $t_{1/2}$, AUC_{∞} , dose-normalized AUC_{∞} (AUC_{∞}/D), apparent volume of distribution (V_z) and apparent volume of distribution at steady-state (V_{ss}) for intravenous and oral doses (Table 1). Oral availability (F) was estimated by dividing AUC_{∞}/D values for the four oral doses by the AUC_{∞}/D values for the two low intravenous doses (10 and 25 mg/kg), where clearance was linear. This

resulted in a comparison of oral and intravenous doses whether the AUC_{∞} values were similar, since the assumption of F calculations is that the drug clearance is the same following intravenous and oral administration. The mean F value was 0.46 for all four doses. The value for the 25 mg/kg oral dose was somewhat lower than that the other doses, and this may represent a true difference or experimental variability. Results from the noncompartmental analysis further confirmed a nonlinear disposition of 1-NITC, noted by the decrease of CL from 2.2 ± 0.9 to 0.8 ± 0.3 L/h/kg and increase of AUC_{∞}/D from 0.5 ± 0.2 to 1.4 ± 0.7 kg \times h/L, when doses increased from 10 to 75 mg/kg.

Pharmacokinetic models I-IV were evaluated to interpret the nonlinear disposition of 1-NITC in rats (Figs. 6-9). Model I, with linear absorption (k_a) from the absorption site to the central compartment, linear elimination rate constant (k) from the absorption site, and linear elimination (CL) from the central compartment, are presented in Fig. 6. Based on this model, plasma concentration-time profiles of both intravenous and oral dosing groups were poorly captured, typically for those data in the elimination phases. In Model II (Fig. 7), a capacity-limited elimination process characterized by Michaelis-Menten kinetics, was used to describe the total clearance of 1-NITC from the central compartment. As a result, the model captured the intravenous data very well, but the data of oral doses were not improved at all, suggesting that both absorption and systemic elimination may be capacity-limited. Based on the information from Model II, Models III and IV incorporated an additional Michaelis-Menten term, i.e. $V'_{max}/(K'_m + A_b)$, nonlinear elimination from the absorption site for Model III (Fig. 8), or nonlinear absorption from the absorption site to the central compartment for Model IV (Fig. 9), respectively. Both models improved the data fitting following intravenous and oral dosing. The

final estimates of V'_{\max} and K_m' were 6.3 ± 2.8 mg/h/kg and 23.6 ± 19.1 mg/kg for Model III and 30.7 ± 0.3 mg/h/kg and 240.7 ± 9.6 mg/kg for Model IV (Table 2). However, the coefficients of variation (CV%) for V'_{\max} (1.1%) and K_m' (4.0%) in Model IV were much lower than those of V'_{\max} (44.9%) and K_m' (80.8%) in Model III. Based on standard goodness-of-fit criteria and the following proposed mechanisms in discussion, Model IV best described nonlinear pharmacokinetics of 1-NITC in rats, and demonstrated nonlinear absorption from the absorption site and nonlinear systemic elimination from the central compartment. As shown in Table 2, the final estimates of the primary parameters were determined as V_c (2.4 ± 0.2 L/kg), V'_{\max} (2.2 mg/h/kg), K_m (0.6 mg/L), k_{12} (1.9 ± 0.2 h $^{-1}$), k_{21} (0.3 h $^{-1}$), k (0.1 h $^{-1}$), V'_{\max} (30.7 ± 0.3 mg/h/kg), and K_m' (240.7 ± 9.6 mg/kg), respectively. The final estimates of the secondary parameters were estimated as V_T (15.4 ± 0.3 L/kg) and CL_D (4.6 ± 0.4 L/h/kg), respectively.

4. Discussion

In exploring new classes of molecules to inhibit multidrug resistance in cancer chemotherapy, we have recently found that some naturally occurring and synthetic ITCs could significantly increase the accumulation of anticancer drugs DNM and VBL in P-gp and MRP1 overexpressed human cancer cells ¹⁰ [Hu and Morris, *J. Pharm. Sci.* in press]. For the twelve ITCs that were evaluated, 1-NITC demonstrated the greatest activities that were comparable with those of the P-gp inhibitor verapamil and MRP1 inhibitor MK-571. In order to optimize dosing regimens of 1-NITC for future *in vivo* studies, the pharmacokinetics of 1-NITC in rats following intravenous and oral administration were investigated in the present study.

After a single intravenous dose of 10, 25, 50 or 75 mg/kg, 1-NITC demonstrated a biexponential disposition in rats (Fig. 2). The kinetic profiles did not demonstrate dose-proportionality (Fig. 3), suggesting that the kinetics of 1-NITC were nonlinear in rats. Noncompartmental analysis (Table 1) of the plasma concentration versus time data further indicated that the disposition of 1-NITC was nonlinear in rats. The compartmental models (Figs. 5-8) were evaluated to simultaneously fit the intravenous and oral data and Model IV (Fig. 8) best described the pharmacokinetics of 1-NITC in rats. As shown in Table 2, the value of V_c (2.4 ± 0.2 L/kg) was 80-fold higher than rat plasma volume (0.03 L/kg)²⁵, suggesting that 1-NITC is a highly protein-bound drug. The protein binding of 1-NITC (10 mg/ml) was determined in our laboratory and was found to be greater than 99% using ultrafiltration. The V_T value for 1-NITC in rats (15.4 ± 0.3 L/kg) was 23-fold higher than rat total body water (0.67 L/kg)²⁵, indicating extensive tissue distribution of 1-NITC in rats likely due to its lipophilicity (Log P 4.34) and preferential tissue binding. This data

is consistent with the observation of Capizoo and Roberts that 1-NITC was widely distributed in all body tissues following oral doses of 50, 100 or 300 mg/kg of ¹⁴C-1-NITC to rats ¹¹.

The nonlinear disposition of 1-NITC in rats is likely due to metabolism of 1-NITC by phases I and II enzymes. We have found that 1-NITC is not detected in unchanged form in the urine ²³, a finding consistent with other studies ²⁶ ¹³. The phase I metabolites of 1-NITC, 1-NA and 1-NIC, were identified in 1988 and 1995, respectively, in rat bile samples ¹⁴ ¹⁵. In our previous and current studies, we found that only 1-NITC and 1-NA were detected in rat plasma and urine, respectively ²³. In addition, we found that 1-NIC is highly reactive and undergoes instant non-enzymatic hydrolysis degradation to 1-NA in aqueous matrix ²³.

The conjugate GS-1-NITC was initially identified in an isolated rat hepatocyte study in which the incubations with 1-NITC resulted in an enhanced efflux rate of GSH from the hepatocytes ¹⁶. However, the authors found that GS-1-NITC formed in the hepatocytes was very unstable, i.e. the $t_{1/2}$ of GS-1-NITC was about 1 min and 95% of GS-1-NITC dissociated at physiological pH to release the original GSH and 1-NITC in 5 min ¹⁶. The discovery of GS-1-NITC conjugate raised interest in the hypothesis that a glutathione conjugate with 1-NITC formed in liver cells is exported into bile where it dissociates to free 1-NITC and GSH ¹⁸. Further studies of Roth and co-workers have supported this hypothesis, though there was huge variability in the results of these two separate studies following the same dose of 100 mg/kg of 1-NITC to rats ²⁰ ²¹. The depletion of hepatic GSH following the administration of buthionine sulfoximide (BSO) prevented the accumulation of 1-NITC in the bile ²⁰. Based on these results, it was proposed that 1-NITC simply diffuses from sinusoidal blood into hepatic parenchymal cells and forms GS-1-

NITC which is further actively transported into bile, where the conjugate dissociates due to its stability and thus releases 1-NITC (and GSH) in high concentrations ²². It is likely that the active transport by Mrp2, which is responsible for the biliary excretion of a variety of endogenous and exogenous organic anions, including many glutathione conjugates ²⁷. This hypothesis is supported by a recent study by Dietrich et al., in which 1-NITC at 5 μ M induced strong apical GSH secretion in the polarized canine kidney MDCK II cells transfected with human MRP2, the Mrp2-deficient TR⁻ rats were protected from 1-NITC (100 mg/kg po) induced cholestasis due to lack of biliary secretion of GS-1-NITC, and BSO treatment (890 mg/kg ip) did not make the TR⁻ rats more sensitive to 1-NITC ²⁸. Enterohepatic cycling of 1-NITC following the biliary elimination of GS-1-NITC and its rapid hydrolysis back to 1-NITC is likely. Although we did not observe any re-entry peaks in the plasma-concentration profiles, the rat does not have a gall bladder so no time delay for the release of 1-NITC or GS-1-NITC into the intestine due to gall bladder emptying would be expected. 1-NITC is a lipophilic compound, so capacity-limited absorption, as suggested by the data fitting was unanticipated. We propose that the capacity-limited absorption reflects the active transport of GS-1-NITC into bile. GS-1-NITC is highly unstable ¹⁶ so undergoes rapid hydrolysis back to 1-NITC, followed by its rapid absorption. This hypothesis will be further evaluated in future studies.

5. Conclusions

In summary, 1-NITC demonstrated nonlinear pharmacokinetics following intravenous and oral administration of a single dose of 10, 25, 50 or 75 mg/kg to rats. Dose-normalized plasma concentration-time profiles were not superimposable for intravenous and oral dosing groups, indicating that the nonlinear disposition of 1-NITC in rats. Results from the noncompartmental analysis further confirmed the nonlinear disposition of 1-NITC, with the decrease of CL and increase of AUC_{∞}/D , as doses increasing from 10 to 75 mg/kg following intravenous administration. A nonlinear two-compartment open model with parallel capacity-limited absorption and capacity-limited elimination from the central compartment best described the pharmacokinetic profiles of 1-NITC following its intravenous and oral administration to rats. We propose that the nonlinear elimination of 1-NITC results from the capacity-limited metabolism of 1-NITC mediated by CYP and GST enzymes; the nonlinear absorption may reflect the rate-limiting transport of GS-1-NITC into bile via the membrane transporter Mrp2, followed by its rapid hydrolysis back to 1-NITC and absorption. The information from this study will be useful in designing dosage regimens of 1-NITC for future investigation as a MDR reversal agent.

Acknowledgements

Financial support for this study was provided by the grants from the Susan G. Komen Breast Cancer Foundation, the Kapoor Charitable Foundation (University at Buffalo) and the U.S. Army Breast Cancer Research Program Contract DAMD17-00-1-0376.

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Table 1. Noncompartmental analysis of data for plasma concentration versus time profile of 1-NITC following intravenous (iv) and oral (po) administration of a single dose of 10, 25, 50 or 75 mg/kg to rats (n = 4 each group).

| Parameters | Units | IV | IV | IV | IV | PO | PO | PO | PO |
|---------------------|--------|--------------|------------|------------|-------------|-------------|------------|-----------|------------|
| | | 75 mg/kg | 50 mg/kg | 25 mg/kg | 10 mg/kg | 75 mg/kg | 50 mg/kg | 25 mg/kg | 10 mg/kg |
| t _{1/2} | h | 8.2 ± 3.6 | 7.9 ± 0.9 | 2.5 ± 0.6 | 12.8 ± 4.3 | 6.1 ± 1.6 | 5.9 ± 0.7 | 5.0 ± 2.4 | 17.0 ± 3.3 |
| AUC _∞ | mg/L*h | 103.7 ± 49.8 | 58.8 ± 8.3 | 11.0 ± 2.9 | 5.2 ± 2.3 | 18.9 ± 12.5 | 10.8 ± 1.8 | 3.4 ± 1.8 | 2.4 ± 1.1 |
| AUC _∞ /D | kg/L*h | 1.4 ± 0.7 | 1.2 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.1 |
| V _{ss} | L/kg | 8.1 ± 2.3 | 9.1 ± 1.3 | 7.8 ± 1.9 | 29.9 ± 20.4 | NA | NA | NA | NA |
| V _z | L/kg | 9.8 ± 5.0 | 9.7 ± 0.8 | 8.8 ± 3.8 | 5.8 ± 4.2 | NA | NA | NA | NA |
| CL | L/h/kg | 0.8 ± 0.3 | 0.9 ± 0.1 | 2.4 ± 0.6 | 2.2 ± 0.9 | NA | NA | NA | NA |

Table 2. Pharmacokinetic parameters for 1-NITC in rats

| Parameters | Units | Estimates | CV (%) |
|------------|----------|-----------|--------|
| V_c | L/kg | 2.4 | 8.6 |
| V_{max} | mg/h/kg | 2.2 | 0.2 |
| K_m | mg/L | 0.6 | 1.3 |
| k_{12} | h^{-1} | 1.9 | 11.6 |
| k_{21} | h^{-1} | 0.3 | 6.4 |
| k | h^{-1} | 0.1 | 10.0 |
| V'_{max} | mg/h/kg | 30.7 | 11.6 |
| K'_m | mg/kg | 241 | 4.0 |
| | | | |
| V_T | L/kg | 15.4 | 1.7 |
| CL_D | L/h/kg | 4.6 | 7.9 |

Figure Legends

Fig. 1. Structures of 1-NITC and metabolites

Fig. 2. Plasma concentration versus time profile of 1-NITC following intravenous administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

Fig. 3. Dose-normalized plasma concentration versus time profile of 1-NITC following intravenous administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

Fig. 4. Plasma concentration versus time profile of 1-NITC following oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

Fig. 5. Dose-normalized plasma concentration versus time profile of 1-NITC following oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

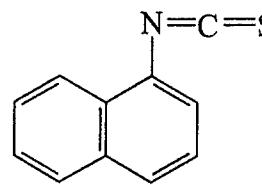
Fig. 6. Model I and fitting of data for plasma concentration versus time profile of 1-NITC following intravenous and oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

Fig. 7. Model II and fitting of data for plasma concentration versus time profile of 1-NITC following intravenous and oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

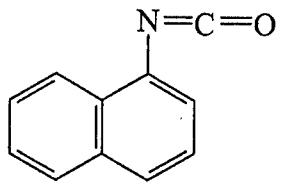
Fig. 8. Model III and fitting of data for plasma concentration versus time profile of 1-NITC following intravenous and oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

Fig. 9. Model IV and fitting of data for plasma concentration versus time profile of 1-NITC following intravenous and oral administration of 10, 25, 50 and 75 mg/kg to rats ($n = 4$ each group).

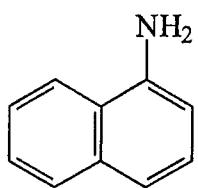
Fig. 1



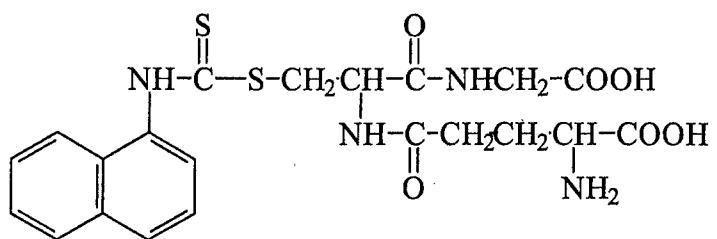
1-NITC



1-NIC



1-NA



GS-1-NITC

Fig. 4

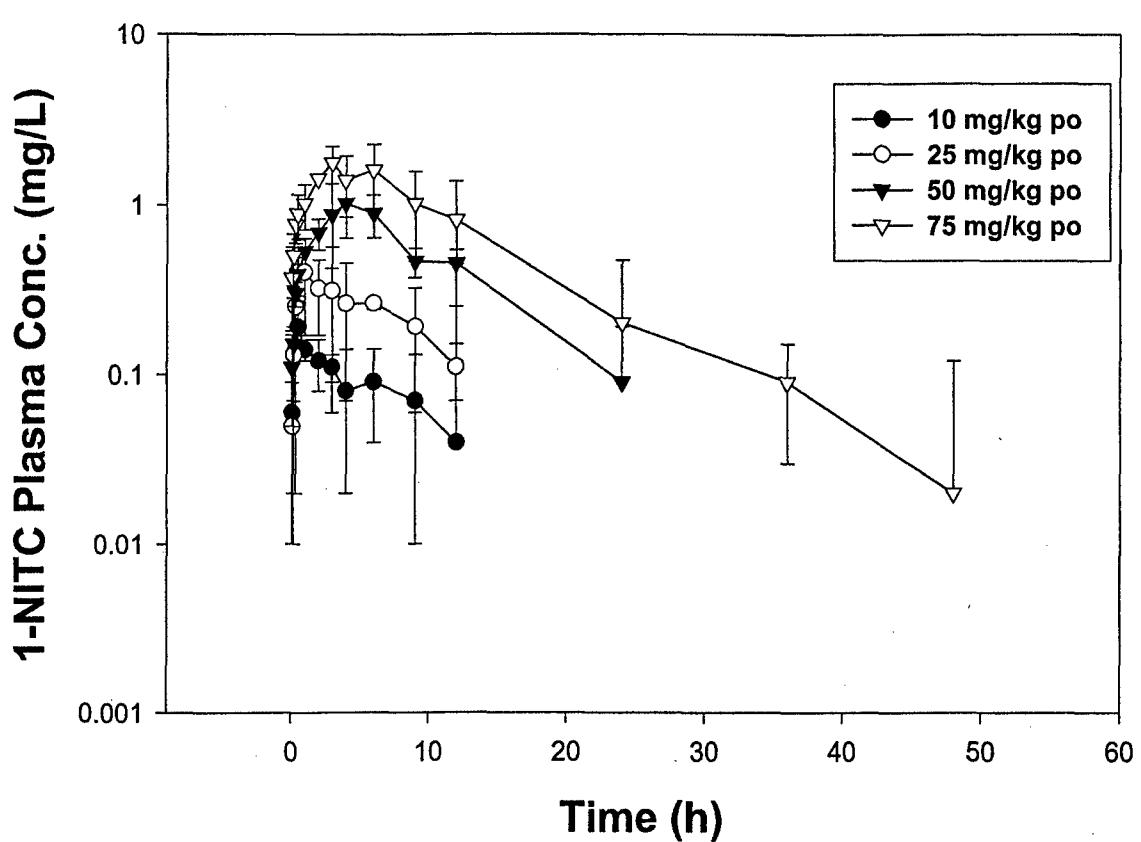


Fig. 5

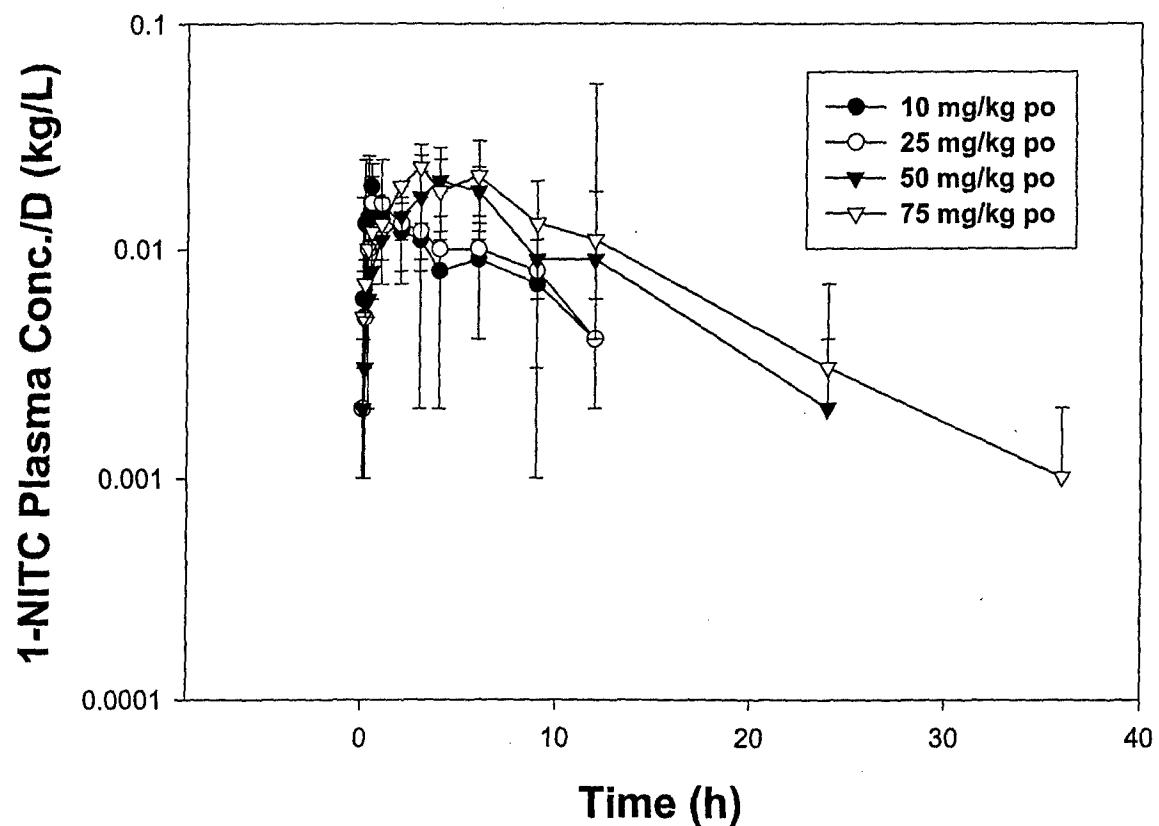


Fig. 6

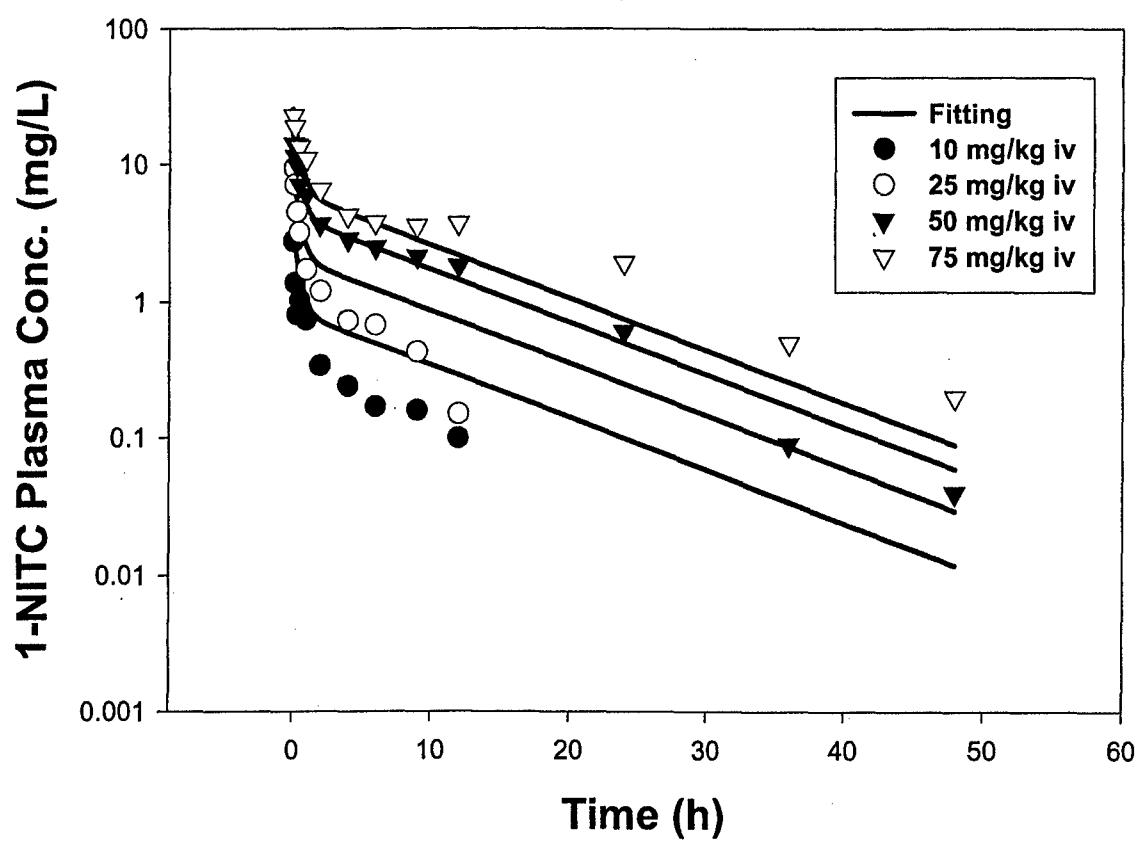
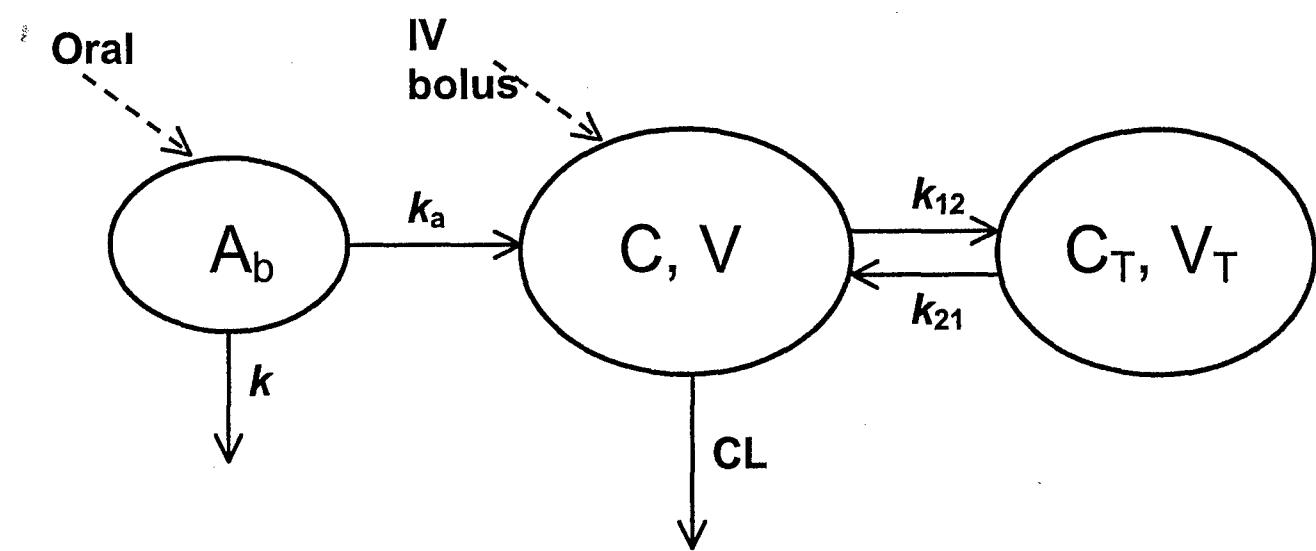
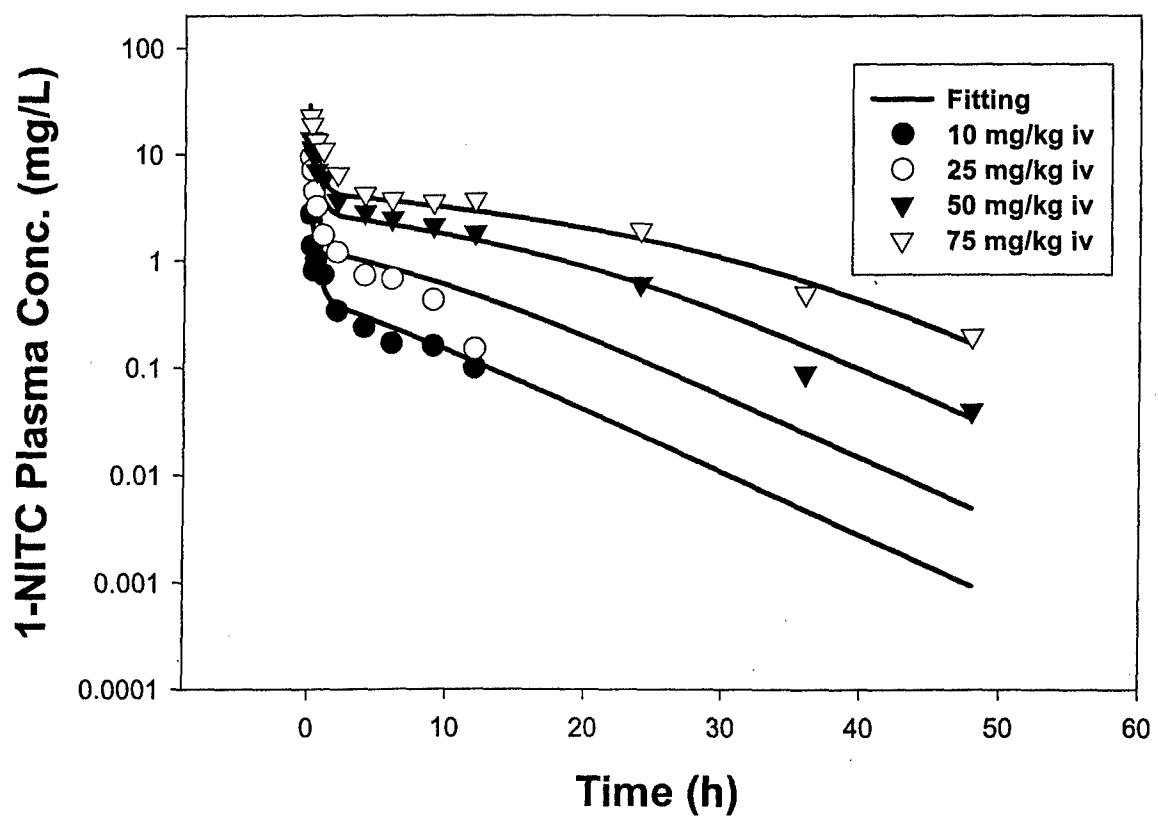
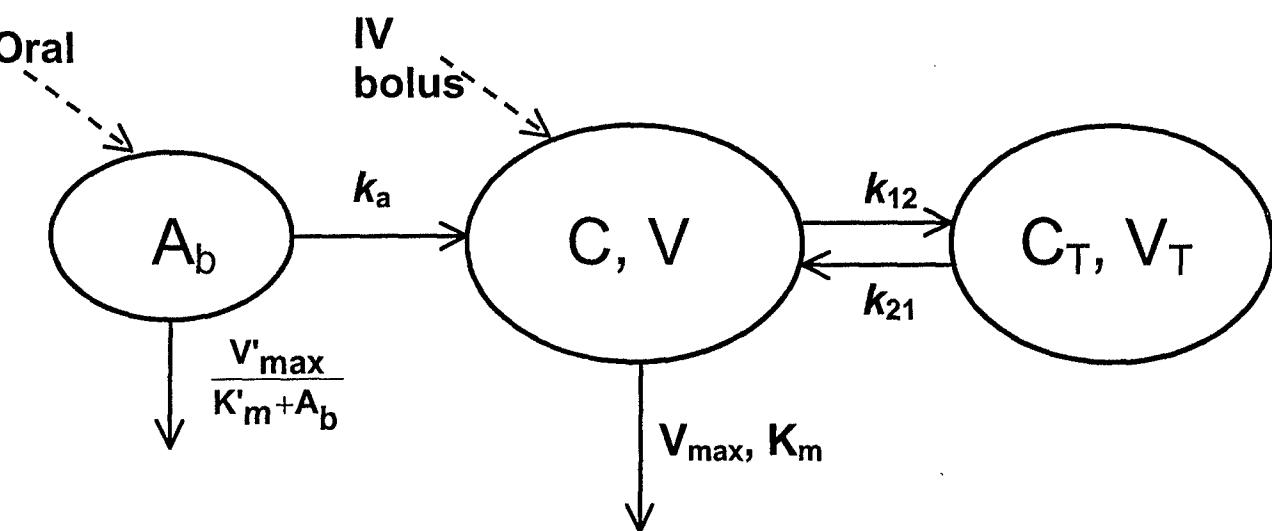


Fig. 8



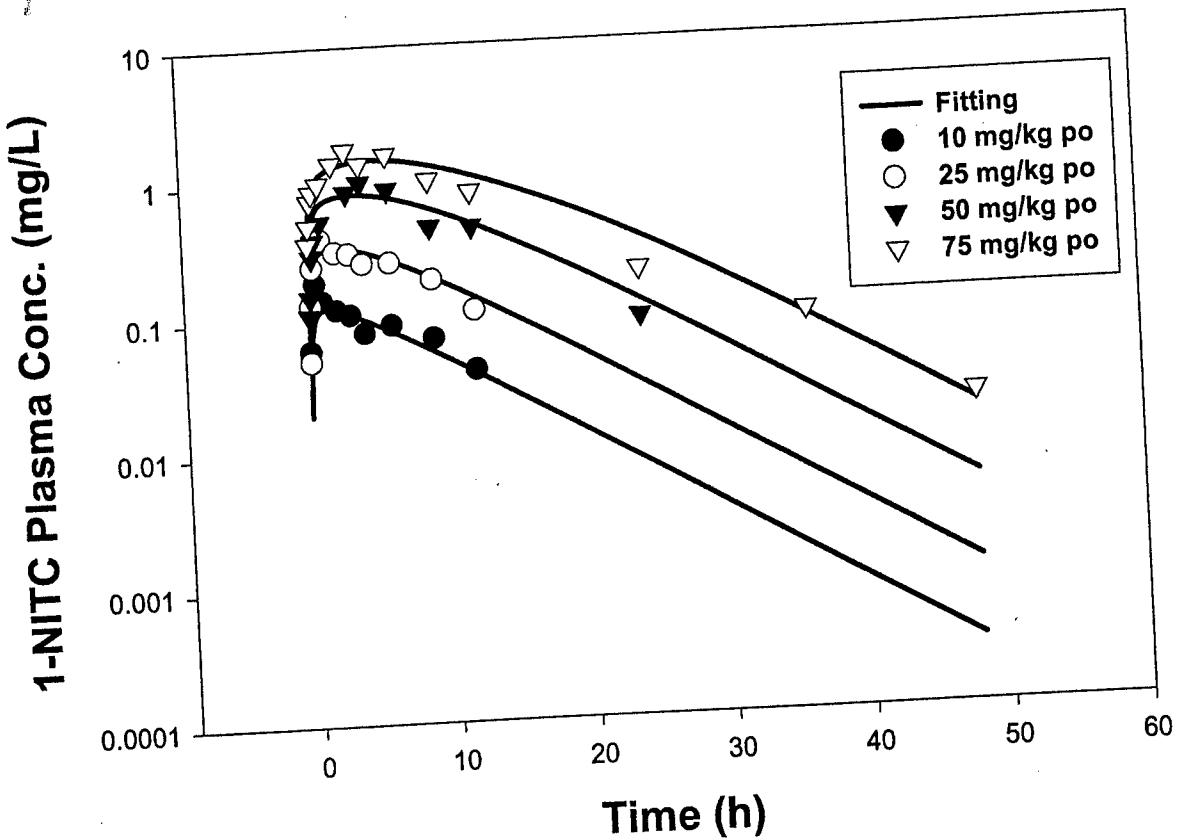


Fig. 9

